University of Alberta

A Study of Peroxisome Biogenesis in the Yeast *Yarrowia lipolytica*

By

Trevor Wayne Brown

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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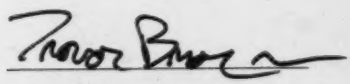

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Abstract

The following thesis describes the use of a negative selection procedure and the resultant identification of a novel *pex* mutant strain, *pex23-1*, in the yeast *Yarrowia lipolytica*. *pex23-1* fails to assemble functional peroxisomes and mislocalizes all peroxisomal matrix proteins investigated to a subcellular fraction enriched for cytosol. Morphologically recognizable peroxisomes are absent in *pex23-1* mutants, which instead accumulate numerous small vesicular structures that are shown to contain both peroxisomal matrix and membrane proteins. Complementation of the *pex23-1* mutant phenotype with a *Y. lipolytica* genomic DNA library resulted in the isolation of the *PEX23* gene encoding the peroxin, Pex23p. Pex23p is a 418-amino acid (47,588 Da), peroxisomal integral membrane protein sequestered from the cytosolic face of peroxisomes. Homology searches have revealed high sequence similarity to two hypothetical proteins of the yeast *Saccharomyces cerevisiae*. Pex23p is present in low amounts in cells grown in glucose-containing medium but is significantly increased in cells grown in oleate-containing medium.

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Dr. Melchior Evers contributed to Figure 3.3 by cloning the *PEX23* gene, performing the restriction map analysis and assisting in subcellular fractionation experiments leading to Figure 3.8 A and C.

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List of Abbreviations

20KgP, 20,000 × *g* pellet
20KgS, 20,000 × *g* supernatant
200KgP, 200,000 × *g* pellet
200KgS, 200,000 × *g* supernatant
AOX, acyl-CoA oxidase
CAT, catalase
CCO, cytochrome *c* oxidase
DTT, dithiothreitol
EDTA, ethylene diaminetetraacetic acid
EMS, ethyl methanesulfonate
ER, endoplasmic reticulum
EST, expressed sequence tag
G6PDH, glucose-6-phosphate dehydrogenase
HRP, horseradish peroxidase
ICL, isocitrate lyase
mPTS, membrane PTS
ORF, open reading frame
PAGE, polyacrylamide gel electrophoresis
PBD, peroxisome biogenesis disorder
PCR, polymerase chain reaction
PMP, peroxisomal membrane protein
PMSF, phenylmethylsulfonyl fluoride
PNS, post-nuclear supernatant
PTS, peroxisome targeting signal
SDS, sodium dodecyl sulfate
SH3, Src homology 3
TCA, trichloroacetic acid
THI, 3-ketoacyl-CoA thiolase

1.0 Introduction

1.1 Organelle biogenesis

Compartmentalization of function is an important evolutionary advantage that distinguishes eukaryotes from prokaryotes. The ability to keep numerous chemical processes separate from one another in membrane-bound compartments has permitted increased metabolic complexity, and the concomitant diversification of cellular functions in eukaryotes. However, since most cellular proteins are not synthesized at their final destinations, the eukaryotic cell has had to develop specific transport mechanisms to direct newly synthesized materials to their sites of function. As well, organelles have to be able to grow as the cell grows, and many may have to be partitioned when the cell divides to permit daughter cells to inherit some of the parental cell material, thereby allowing for transmission of functional capacity. These are all aspects of organelle biogenesis that have been studied quite extensively for organelles such as the endoplasmic reticulum (ER) (Blobel and Dobberstein, 1975; Blobel, 1980; Sabatini *et al.*, 1982), the chloroplast (Robinson and Klösgen, 1994), and the mitochondrion (Hartl *et al.*,1989; Schatz and Dobberstein, 1996). In contrast, only recently have advances been made in understanding the biogenesis of peroxisomes. Much remains to be understood about how the cell targets proteins to peroxisomes, how it regulates peroxisomal growth, and what factors are required for peroxisome assembly.

1.2 Peroxisomes: What are they?

Peroxisomes belong to the microbody family of organelles, which includes the glyoxysomes of plants and the glycosomes of trypanosomes (Lazarow and Fujiki, 1985).

Peroxisomes were first identified as microbodies by Rhodin in the 1950s while performing ultrastructural studies on mouse kidney cells (Rhodin, 1954). The term "peroxisome" was coined by de Duve who, with his colleagues, pioneered the initial biochemical characterization of this organelle in rat liver. de Duve defined a peroxisome as an organelle containing at least one H_2O_2-forming oxidase and catalase to decompose it (de Duve and Baudhuin, 1966). Thereafter, related organelles were discovered in plants (glyoxysomes) (Breidenbach and Beevers, 1967) and in trypanosomes (glycosomes) (Opperdoes and Borst, 1977). Peroxisomes are now known to be found in most eukaryotic cells (Hruban *et al.*, 1972), where they carry out a diverse set of functions depending on cell and tissue type (see below).

Morphologically, peroxisomes are usually spherical, ranging from 0.2 μm to 1 μm in diameter, often contain a paracrystalline core, and are bounded by a single unit membrane. Peroxisomes do not contain DNA (Kamiryo *et al.*, 1982) and are of high equilibrium density in sucrose (~1.23 g/cm ^{3}) at maturation, as compared to other organelles such as mitochondria and microsomes.

1.3 Functions of peroxisomes

Since the initial discovery of catalase in peroxisomes, which serves to remove toxic peroxide byproducts (de Duve and Baudhuin, 1966), a number of important metabolic functions have been attributed to peroxisomes. The most notable function is the β-oxidation of fatty acids. This process appears to be ubiquitous, occurring in the peroxisomes of animals (Lazarow and de Duve, 1976), plants (Beevers *et al.*, 1969) and yeasts (Tanaka *et al.*, 1982). Peroxisomes have also been shown to be involved in the

early reactions of plasmalogen biosynthesis (Hajra *et al.*, 1979; Hajra and Bishop, 1982), an ether phospholipid that is abundant in nervous tissue such as myelin. They have also been shown to be involved in the early stages of cholesterol biosynthesis (Keller *et al.*, 1985, 1986; Krisans, 1996), in alcohol oxidation (Veenhuis *et al.*, 1983), as well as in many other metabolic functions (reviewed in Lazarow and Moser, 1995).

1.4 Protein import into peroxisomes

As described above, the peroxisome is the site of numerous metabolic pathways for the cell and, hence, requires the correct subset of enzymes for proper functioning. Since peroxisomes do not contain any DNA all peroxisomal proteins, once synthesized, must be transported to the organelle (Lazarow and Fujiki, 1985). As with other organelles, the cell has developed mechanisms to traffic proteins specifically to the peroxisome.

Proteins destined for a specific cellular compartment are proposed to carry structural signals that are recognized by receptor structures on the target organelle (Blobel, 1980; Sabatini *et al.*, 1982). Peroxisomal matrix proteins have been found to be targeted to peroxisomes by one of two peroxisome targetin signals (PTS). PTS1, first discovered in firefly luciferase (Gould *et al.*, 1987, 1989) is a conserved tripeptide located at the extreme C-terminal end of a protein. It consists of the sequence Ser-Lys-Leu or conserved variants of these residues. The majority of peroxisomal matrix proteins contain a PTS1 (Gould *et al.*, 1989; Aitchison *et al.*, 1991; Swinkels *et al.*, 1992; Motle *et al.*, 1995; Elgersma *et al.*, 1996). A second PTS, PTS2, was also identified and is a conserved N-terminal nonapeptide (Arg/Lys)(Leu/Val/Ile) Xaa_5 (His/Gln)(Leu/Ala). It is

used by a smaller subset of matrix proteins, most notably the β-oxidation enzyme 3-ketoacyl-CoA thiolase (Swinkels *et al.*, 1991; Glover *et al.*, 1994b), as well as by plant glyoxysomal malate dehydrogenase (Gietl *et al.*, 1994) and *Hansenula polymorpha* amine oxidase (Faber *et al.*, 1995). A striking feature that emerged as a result of the identification of these PTSs was their evolutionary conservation from yeasts to humans (Gould *et al.*, 1990).

Much less is known about the targeting of membrane proteins to peroxisomes. A membrane PTS (mPTS) has been identified in two proteins to date: *Candida boidinii* PMP47 (McCammon *et al.*, 1994; Dyer *et al.*, 1996) and *Pichia pastoris* Pex3p (Wiemer *et al.*, 1996). The overall similarity between the two consensus mPTSs is low, but the do share a common charged stretch of five basic amino acids that are essential for targeting (KIKKR in *Cb*PMP47 and RNKKK in *Pp*Pex3p). Both mPTSs have been shown to be sufficient to target fusion proteins to the peroxisomal membrane and render them resistant to alkaline carbonate extraction (McCammon *et al.*, 1994; Dyer *et al.*, 1996). In addition to a weak overall similarity, their locations within the proteins also vary. The mPTS in *Cb*PMP47 is located in a 20-amino acid loop between two transmembrane domains (Dyer *et al.*, 1996), while *Pp*Pex3p lies in the N-terminal 40-amino acid region (Wiemer *et al.*, 1996).

Other peroxisomal proteins have less well defined PTSs, yet are still able to be targeted to the organelle. It has been shown that proteins without a PTS can interact with PTS-containing proteins and effectively "piggy back" their way into the peroxisome (Glover *et al.*, 1994a; McNew and Goodman, 1994). In addition, these experiments demonstrated that unlike protein import into mitochondria or chloroplasts, proteins

imported into the peroxisome need not be unfolded during the import and that multi-subunit proteins can be imported without dissociation (Glover *et al.*, 1994a; Walton *et al.*, 1995).

1.5 Human disorders of peroxisome biogenesis

Emphasizing the need to study the mechanisms of protein trafficking to peroxisomes is the existence of several classes of autosomal recessive human disorders in which peroxisomes fail to assemble properly and do not import critical matrix proteins. The result is impaired peroxisomal function, which leads to developmental defects and, most often, death in early infancy (reviewed in Lazarow and Moser, 1995; Subramani 1998). These peroxisome biogenesis disorders (PBD) are classified into three groups. Group A comprises Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease. PBD patients of Group A display the most severe phenotypes. They are characterized by severe neurological and hepatic dysfunction, mental retardation, craniofacial abnormalities, and hypotonia. Patients have very high levels of phytanic acid and very-long chain fatty acids in the blood and cannot synthesize plasmalogens, a very important class of phospholipids in nervous tissue (Schutgens *et al.*, 1986). Survival beyond infancy for Group A patients is extremely rare. Peroxisomes in the cells of group A patients are unable to import proteins targeted by either PTS1 or PTS2, and thus most of their peroxisomal metabolic functions are impaired.

Rhizomelic chondrodysplasia punctata, a Group B disorder, is characterized by severe growth defects, rhyzomelia, cataracts, proximal limb shortening and ichthyosis (Lazarow and Moser, 1995). These patients have normal blood levels of very-long chain

fatty acids but have increased levels of phytanic acid and also lack plasmalogens. Unlike Group A patients, peroxisomes in the cells of Group B patients are deficient in the import of PTS2 proteins only and have normal PTS1 import.

The third class of PBDs, Group C disorders, do not involve a general mislocalization of peroxisomal matrix proteins. Rather, the milder symptoms associated with this class of disorder are the result of mutations in single genes which affect the function or targeting of individual peroxisomal enzymes. Examples of Group C disorders are X-linked adrenoleukodystrophy, acatalasaemia (catalase deficiency) and adult Refsum disease.

Peroxisomes in cells of Group A PBD patients were originally thought to be absent (Goldfischer *et al.*, 1973), but later studies revealed the existence of peroxisomal structures in cell lines from several patients (Santos *et al.*, 1988). In fact, studies showed that while matrix proteins containing PTS1 or PTS2 were mislocalized to the cytosol in these cell lines, peroxisomal membrane proteins (PMP) were correctly targeted. This finding has also been corroborated for certain peroxisome assembly mutants in yeasts. This indicates that there are probably separate protein import pathways for peroxisomal matrix and membrane proteins (Slawecki *et al.*, 1995).

1.6 Yeast as a model system to study the mechanisms of peroxisome biogenesis

Early investigations into the mechanisms of peroxisome assembly involved the use of cultured cell lines derived from affected PBD patients. Somatic cell hybridization studies revealed the existence of several complementation groups among the PBD cell lines, indicating that at least several proteins (peroxins) are involved in peroxisomal

biogenesis (Brul *et al.*, 1988; Roscher *et al.*, 1989). However, attempts to clone the defective genes in these cells were unsuccessful due to the technical difficulties inherent in working with somatic human cell lines. Accordingly, most of the advances in our understanding of peroxisome protein import and most of the genes identified to date have come initially from yeast model systems.

Many features of yeast make it ideally suited for studying the mechanisms of peroxisome biogenesis. Unlike peroxisomes of animals, yeast peroxisomes are the only sites of fatty acid β-oxidation in the cell and therefore make it a simpler organism in which to study this process (Tanaka *et al.*, 1982). Interestingly, peroxisomes of yeast and their proteins undergo tremendous induction when grown on certain "peroxisome-requiring" carbon sources such as oleate (Veenhuis *et al.*, 1987; Kunau *et al.*, 1988) or methanol (Fukui *et al.*, 1975). The presence of large and numerous peroxisomes facilitates their isolation for biochemical studies and morphological analysis. Another fortuitous feature is that peroxisomes in yeast are repressible and even dispensable when grown on carbon sources such as glucose, which do not require the metabolizing functions of peroxisomes for conversion to a usable energy source. This makes possible the creation of yeast peroxisome assembly (*pex*) mutants (Distel *et al.*, 1996) that can be easily maintained in culture, thereby facilitating the cloning of genes required for peroxisome assembly. Another important feature of yeast model systems is that their genetics are extremely well understood. There are numerous molecular tools available to manipulate genetic elements, introduce exogenous genes or delete endogenous ones, all making yeast an ideal system in which to piece together the mechanisms of peroxisome biogenesis.

1.7 *Yarrowia lipolytica*

Many different yeast species are currently being used as model systems for peroxisome biogenesis. For our studies (and in this thesis) we use the dimorphic yeast species *Yarrowia lipolytica*. *Y. lipolytica* grows extremely well on fatty acid carbon sources (Kamiryo *et al.*, 1979), unlike *Saccharomyces cerevisiae* or *P. pastoris*. A successful rapid selection system for *pex* mutants has been developed for *Y. lipolytica* (Nuttle *et al.*, 1993) and has led to the isolation of several *Y. lipolytica pex* mutants and their complementing *PEX* genes. This species has been thoroughly characterized genetically and has numerous plasmid vectors available (Barth and Gaillardin, 1996), as well as an efficient means of transformation (Davidow *et al.*, 1987; Gaillardin and Ribet 1987) that greatly facilitates the cloning of new genes. The physiology and biology of the species are nicely reviewed (Barth and Gaillardin, 1997).

1.8 Genetic screens

The most common (and most successful) method to dissect the components involved in the peroxisome assembly pathway is through the use of yeast genetic screens. Yeast are mutagenized in order to make *pex* mutants defective in peroxisome biogenesis that are isolated through either positive or negative selection procedures. These mutants are then rescued by transformation with a genomic library in order to complement the mutant phenotype. The complementing gene is isolated and its gene product characterized (Elgersma and Tabak, 1996). This method has proven highly successful, as 23 individual *PEX* genes have been isolated to date (based on currently assigned *PEX* gene numbers) from a variety of yeast species, including *S. cerevisiae* (Erdmann *et al.*,

1989), *P. pastoris* (Gould *et al.*, 1992), *H. polymorpha* (Creg *et al.*, 1990) and *Y. lipolytica* (Nuttle *et al.*, 1993).

1.9 Evolutionary conservation of *PEX* genes

A revealing aspect from the identification of all the *PEX* genes is their remarkable conservation in evolution, not only among *PEX* genes from different yeast species, but with human *PEX* genes as well. In fact, most human *PEX* genes have been identified through homology-based cloning strategies using the Expressed Sequence Tag (EST) database (Dodt *et al.*, 1996; reviewed in Subramani, 1997). The initial identification of yeast *PEX* genes has led directly to the identification of the genes affected in over 90% of the known human PBDs (Chang *et al.*, 1999). Reinforcing the suitability of yeast model systems to understand peroxisome biogenesis in humans is the fact that for every human *PEX* gene identified so far, there is a corresponding yeast homolog. This fact underlines the importance of continuing the search for novel yeast *pex* mutants.

1.10 Current models of peroxisome biogenesis

Peroxisomes were originally proposed to form by budding from extensions of the ER (Novikoff and Shin, 1964). This view was based mainly on morphological data demonstrating peroxisomes and ER in proximity to one another and the appearance of continuity (tails) between their membranes, which was assumed to link the two (Novikoff and Shin, 1964). This model was later superceded by the growth and division model of Lazarow and Fujiki (1985), which states that new peroxisomes arise exclusively by budding and fission of pre-existing peroxisomes. Evidence in support of this model came

from the demonstration that the peroxisomal proteins investigated were synthesized in the cytosol and then post-translationally imported into the peroxisome (reviewed in Lazarow and Fujiki, 1985). Also, studies showed that peroxisomal matrix proteins lacking PTSs were mislocalized to the cytosol, not the ER (Gould *et al.*, 1989; Elgersma *et al.*, 1996). There was also evidence showing that PMPs were targeted to peroxisomes from the cytosol (Fujiki *et al.*, 1984).

Of late, however, our knowledge of the biogenesis of peroxisomes has increased dramatically due to the increasing numbers of *PEX* genes being identified. Several recent studies have suggested that there may be a role for the ER in peroxisome biogenesis. Titorenko and coworkers have demonstrated that mutations in certain secretory genes, as well as in two *PEX* genes (*PEX1* and *PEX6*), can result in the accumulation of the PMPs Pex2p and Pex16p in the ER (Titorenko *et al.*, 1997). Another PMP, Pex15p, when overexpressed causes a proliferation of ER membranes and itself accumulates in the ER (Elgersma *et al.*, 1997). However, whether the ER is a necessary transport pathway for the biogenesis of peroxisomes still remains to be proven.

The ever increasing number of new peroxins is also helping to unravel the mechanisms of protein import into the peroxisome. Early screens for yeast *pex* mutants revealed strains defective in both PTS1- and PTS2-mediated import, as well as strains defective in import by only one or the other pathway. Mutants defective only in the import of PTS1 proteins led directly to the identification of the PTS1 receptor, Pex5p, in *P. pastoris* (McCollum *et al.*, 1993; Terleck *et al.*, 1995). Subsequently, paralogs of Pex5p have been identified in several other organisms (van der Leij *et al.*, 1993; Szilard *et al.*, 1995; van der Klei *et al.*, 1995), including humans (Dodt *et al.*, 1995).

Likewise, yeast mutants defective solely in the import of PTS2 proteins led to the identification of the PTS2 receptor, Pex7p, in several organisms (Marzioch *et al.*, 1994; Rehling *et al.*, 1996; Elgersma *et al.*, 1998), including humans (Braverman *et al.*, 1997).

Although it has been shown that PTS1 and PTS2 receptors mediate separate targeting pathways, the existence of mutants compromised in both pathways indicates that at some point, the pathways converge. The recent cloning of the peroxins Pex13p and Pex14p has shed light on this issue. Pex13p is an integral PMP with Src homology 3 (SH3) domains and has been shown to be a peroxisomal docking site for Pex5p (PTS1 receptor) (Elgersma *et al.*, 1996) and also to interact (directly or indirectly) with Pex7p (PTS2 receptor) (Girzalsky *et al.*, 1999). Pex14p is also an integral PMP and has been shown to interact with both Pex5p and Pex7p, in addition to Pex13p and itself (Brocard *et al.*, 1997; Will *et al.*, 1999). Pex14p is thought be the point of convergence for the two PTS pathways, which presumably would then share a common translocation machinery. Deletions of either *PEX13* or *PEX14* in yeast results in the loss of import by both PTS pathways (Gould *et al.*, 1996; Albertini *et al.*, 1997).

Impeding the progress towards a clear cut model for protein import into peroxisomes is the dispute regarding the subcellular locations of the PTS1 and PTS2 receptors. Varying reports have placed the PTS1 receptor as being primarily cytosolic (Gould *et al.*, 1996), associated with the outer face of the peroxisome (Terleck *et al.*, 1996) or completely peroxisomal (Szilard *et al.*, 1995). Similarly baffling is the subcellular localization of the PTS2 receptor. It has been reported as being cytosolic (Marzioch *et al.*, 1994) or entirely peroxisomal (Zhang and Lazarow, 1996).

In light of these differences, a model has been proposed that attempts to reconcile the different localizations of these receptors. This "extended shuttle" hypothesis (Rachubinski and Subramani, 1995; Dodt and Gould, 1996; Erdmann *et al.* , 1997) suggests that the import receptors, Pex5p and Pex7p, bind their respective matrix proteins in the cytosol, dock to specific proteins at the peroxisomal membrane, enter the peroxisomal matrix where they release their cargo and then shuttle back to the cytoplasm. The existence of Pex13p and Pex14p as docking factors for the PTS receptors fits this model. The prior demonstration that the peroxisome is capable of translocating folded and oligomeric proteins (Glover *et al.*, 1994a; McNew and Goodman, 1994) supports this model as well.

1.11 Thesis overview

This thesis reports investigations into the mechanisms of peroxisome biogenesis in the yeast *Yarrowia lipolytica*. The intention of this project was to use a genetic screen to identify a novel yeast mutant impaired in the assembly of peroxisomes, with the goal of eventually cloning a novel *Y. lipolytica PEX* gene involved in this pathway.

In this thesis, I report the isolation and analysis of a novel mutant of peroxisome assembly, *pex23*, of *Y. lipolytica*, as well as of its complementing gene, *PEX23*, and its encoded peroxin, Pex23p.

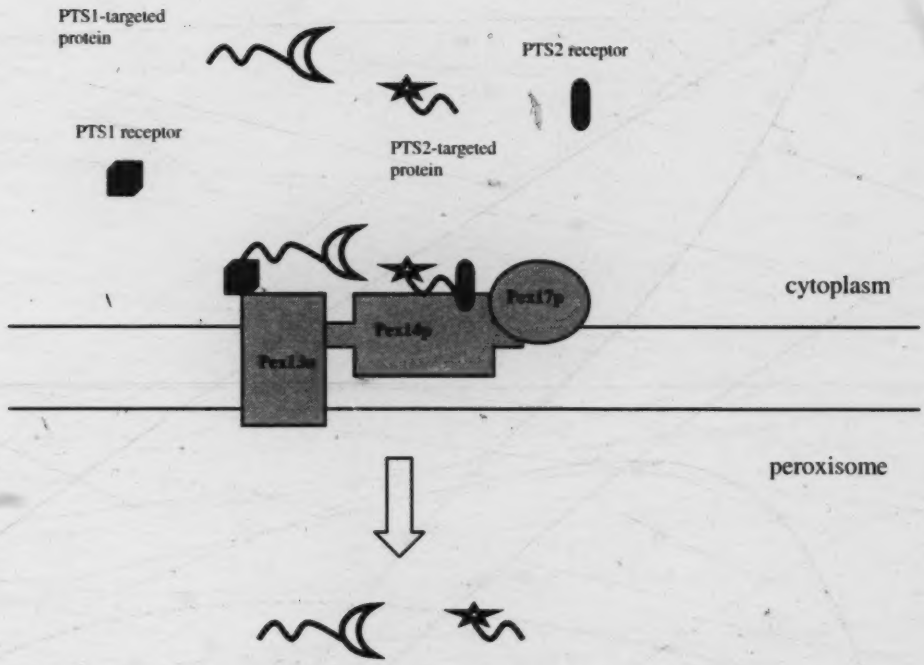

Figure 1.1. Model of how Pex13p, Pex14p and Pex17p may act as a docking complex for the PTS1 receptor, Pex5p, and the PTS2 receptor, Pex7p, at the peroxisomal membrane.

2.0 Materials and Methods

2.1.0 Materials

Some key materials and their corresponding suppliers:

2.1.1 Reagents and chemicals

agar	Difco
agarose	FMC / Gibco
albumin, bovine serum (BSA) fraction V	Roche
L-amino acids	Sigma
ampicillin	Sigma
antipain dihydrochloride	Roche
aprotinin	Roche
$\alpha[^{32}P]$-dATP	Amersham Pharmacia
benzamidine hydrochloride	Sigma
BioRad protein assay dye reagent	BioRad
Brij-35 (polyoxyethylene 23-lauryl ether)	Sigma
chymostatin	Sigma
Coomassie Brilliant Blue (R-250)	Gibco
cytochrome c (horse heart)	Sigma
dithiothreitol (DTT)	Roche
ethylene diaminetetraacetic acid (EDTA)	Sigma
ethyl methanesulfonate (EMS)	Sigma
formamide	Fisher
formaldehyde	ICN
5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-gal)	Sigma
D-glucose	BDH
glutaraldehyde	EMS
hydrogen peroxide (H_2O_2)	Fisher
leupeptin	Roche
malic acid	Sigma
2-(N-morpholino) ethane sulfonic acid	Sigma
nitrocellulose (Hybond-C and Transblot)	Amersham Pharmacia / BioRad
oleic acid	Fisher
osmium tetroxide	EMS
ovalbumin	Sigma
pepstatin A	Sigma
peptone	Difco
phenol, buffer saturated	Gibco
potassium permanganate	Fisher
sodium dodecyl sulfate (SDS)	Sigma
sorbitol	BDH

sucrose	BDH
trichloroacetic acid (TCA)	BDH
Tris (Tris(hydroxymethyl)aminomethane)	Roche
Triton X-100	Sigma
Tween 20 (polyoxyethylene monolaurate)	Sigma
Tween 40 (polyoxyethylene sorbitan palmitate)	Sigma
uranyl acetate	J.B. EM
urea	ICN
yeast extract	Difco
yeast nitrogen base without amino acids	Difco

2.1.2 Enzymes

calf intestinal phosphatase (CIP)	NEB
DNA ligase, T4	Gibco
DNA polymerase, Klenow fragment	NEB
DNA polymerase, T4	NEB
DNA polymerase, T7 sequenase (version 1.0)	USB
restriction endonucleases	NEB / Roche / Promega
ribonuclease A (RNase A)	Roche
trypsin	Roche
Zymolyase 100T	ICN

2.1.3 Experimental kits

QIAprep Spin Miniprep Kit	QIAGEN
QIAquick Gel Extraction Kit	QIAGEN
Random Hexanucleotide Primer Labeling Kit	Roche
ECL Direct Nucleic Acid Labeling and Detection System	Amersham Pharmacia
ECL Western Blotting Detection Reagents	Amersham Pharmacia
ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit	PE Applied Biosystems
DNA UltraFast Cleavage and Deprotection Kit	Beckman

2.1.4 Plasmids

i) *Escherichia coli*

pGEM5Zf (+)	Promega
pGEM7Zf (+)	Promega
pGEM–T	Promega
pSP73	Promega
pBluescript SK (II)	Stratagene

ii) *Y. lipolytica* shuttle vectors

pINA443	Dr. Claude Gaillardin (INRA, Thiverval-Grignon France)
pINA445	Dr. Claude Gaillardin (INRA, Thiverval-Grignon, France)
pTC3 (pTEC)	Ms. Jennifer Smith (University of Alberta, Canada)
pSU	Mr. Arjunah Thiagarajah (University of Alberta, Canada)
p*PEX23*-Myc	This stud
p*PEX23*-TH	This stud

2.1.5 Molecular size markers

1 kb ladder	Gibco
Prestained Markers for SDS-PAGE (6.5, 16.5, 25, 32.5, 47.5, 62, 83, 175 kDa)	NEB

2.1.6 Antibodies

i) Primary antibodies

Mouse monoclonal antibody 9E10 specific for c-Myc p67 of human origin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibod A-9521 specific for *S. cerevisiae* glucose-6-phosphate dehydrogenase (G6PDH) was from Sigma.

ii) Secondary antibodies

The following table lists all the secondary antibodies used in this thesis and the sources from which they were obtained. Secondary antibodies were either conjugated to horseradish peroxidase (HRP) for immunoblotting or to a fluorescent dye molecule (FITC (fluorescein) or TRITC (rhodamine)) for indirect immunofluorescence.

sheep anti-mouse-HRP conjugated	Amersham Pharmacia
donkey anti-rabbit-HRP conjugated	Amersham Pharmacia
goat anti-guinea pig-HRP conjugated	Sigma
donkey anti-mouse-FITC conjugated	Jackson
donkey anti-rabbit-FITC conjugated	Jackson
donkey anti-guinea pig-FITC conjugated	Jackson
donkey anti-mouse-TRITC conjugated	Jackson
donkey anti-rabbit-TRITC conjugated	Jackson
donkey anti-guinea pig-TRITC conjugated	Jackson

iii) Laboratory-derived antibodies

All other antibodies used during the studies reported in this thesis were raised by others or obtained from outside sources. Anti-SKL antibodies were raised in rabbit against the peptide NH_2-CRYHLKPLQSKL-COO_2H linked to keyhole limpet hemocyanin, as described (Aitchison *et al*., 1992). Antisera to *Y. lipolytica* peroxisomal thiolase (guinea pig) and isocitrate lyase (ICL) (rabbit), as well as anti-Pex2p (Pay5p) (guinea pig) were raised as described (Eitzen *et al.*, 1996). Anti-Pex16p antibodies were raised in guinea pig, as described (Eitzen *et al.*, 1997). Antiserum to the *Y. lipolytica* peroxisomal matrix protein acyl-CoA oxidase (AOX), raised in rabbit, was a kind gift of Dr. Jean-Marc Nicaud (INRA, Thiverval-Grignon, France).

2.2.0 DNA manipulation and detection

2.2.1 Molecular cloning

All molecular cloning was performed essentially as described (Maniatis *et al.*, 1982; Ausubel *et al*., 1989). *E. coli* DH5α cells were grown at 37°C in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) and contained 100 µg ampicillin/ml in

order to maintain selective pressure for plasmids. Restriction digests and DNA modifications were performed according to manufacturers' instructions.

2.2.2 *Isolation of nucleic acids*

i) E. coli

Plasmid DNA was isolated from small-scale bacterial cultures according to the alkaline lysis mini-prep procedure (Ausubel *et al.*, 1989) or by using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions.

ii Y. lipolytica

Total genomic and/or plasmid DNA was isolated from *Y. lipolytica* strains using the glass bead disruption technique (Ausubel *et al*., 1989) in breakage buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2% (w/v) Triton X-100, 1% SDS). Cells were grown overnight in 10 ml cultures of appropriate medium (rich or selective), washed three times with water and then resuspended in breakage buffer. Glass beads were added, tubes were vortexed three times for 1 min each, and then cell debris was removed by centrifugation for 5 min at maximum speed in a microfuge. Nucleic acids were separated from proteins with two phenol/chloroform/isoamyl alcohol (25:24:1) extractions and one chloroform/isoamyl alcohol (24:1) extraction. Total nucleic acids were precipitated by the addition of 2.5 volumes of absolute ethanol, pelleted by centrifugation in a microfuge for 4 min at maximum speed, and subsequently washed twice with 70% ethanol to remove contaminating salts. The nucleic acid pellet was then resuspended in approximately 50 µl of TE/RNase A (10 mM Tris-HCl, pH 8.0, 1 mM

EDTA, 20 µg RNase A/ml) and incubated at room temperature overnight to allow for RNA digestion and pellet dissolution. Plasmid DNA was recovered by subsequent electroporation into *E. coli*.

2.2.3 Electroporation of E. coli

High-efficiency electroporation-competent *E. coli* cells were prepared according to Maniatis *et al.* (1982). Freshly grown *E. coli* were used to inoculate 50 ml of S.O.B. medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, pH 7.0) and were grown overnight at 37°C. Cultures were then diluted into 500 ml S.O.B. and grown for 2 to 3 h at 37°C until the OD_{550} was approximately 0.8. Cells were harvested by centrifugation and washed twice with 500 ml of sterile, ice-cold 10% (v/v) glycerol. The pellet was then resuspended in 10% (v/v) glycerol to a final volume of 2 ml, and 20 µl aliquots were subjected to electroporation using a Gibco Cell-Porator at 370 V and a resistance of 4 Ω. Cells were then diluted to 1 ml with LB medium, grown for 1 h at 37°C and plated onto LB-agar plates containing ampicillin.

2.2.4 Isolation of DNA fragments and PCR products

DNA fragments produced in the course of molecular cloning and polymerase chain reaction (PCR) products were first separated by electrophoresis using SeaKem GTG agarose (FMC, Rockland, ME) in 1 × TBE buffer (89 mM Tris-borate, pH 8.0, 89 mM boric acid, 2 mM EDTA) containing 5 µg ethidium bromide/ml. Gels were run at a 8-10 V/cm of gel. Prior to loading, samples were mixed with 6 x Gel Dye (0.01% bromophenol blue, 0.01% xylene cyanol, 5% (w/v) glycerol). The bands of interest were

visualized with ultraviolet illumination and excised from the gel. The QIAquick gel extraction kit was used to recover the DNA. Gel fragments were dissolved by a chaotropic agent and heating at 50°C for 10 min, according to the manufacturer's protocol. The solution was loaded onto a QIAquick column and spun in a microfuge to bind the DNA. The bound DNA was washed of contaminating salts and eluted using 30 to 50 µl of 10 mM Tris-HCl (pH 7.5) and stored at -20°C until needed.

2.2.5 *Labelling of DNA probes*

For all Southern blot analyses or colony hybridization experiments, labelled DNA probes were made using 50 to 200 ng of DNA fragments. Radiolabelled probes were made using the Random Hexanucleotide Primer Labeling Kit. Enzymatic probes using HRP conjugated to DNA were made using the ECL Direct Nucleic Acid Labeling and Detection System. In both cases, DNA was denatured by boiling for 5 min followed by rapid cooling on ice. The denatured DNA was then either radiolabelled using random primers and DNA polymerase to incorporate $\alpha[^{32}P]$-dATP or enzymatically labelled with HRP using the cross-linker, glutaraldehyde. Unincorporated radiolabel was removed by size separation on a Sephadex G50 microcentrifuge spin column (Maniatis *et al*., 1982). Specific activities of radiolabelled probes were determined by liquid scintillation counting.

2.2.6 *Southern blot analysis*

Genomic DNA to be analyzed was digested overnight with appropriate restriction enzymes. About 1 µg of digested DNA was loaded per lane of an agarose gel and

subjected to electrophoresis. The gel was exposed to ultraviolet light for 5 min in order to nick the DNA and then placed in denaturing solution (1.5 M NaCl, 0.5 M NaOH) with gentle shaking for 30 min at room temperature. The gel was then placed in renaturing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) again with gentle shaking for 30 min at room temperature. This renaturation step was repeated. DNA was transferred to nitrocellulose essentially as described by Southern (1975) and modified by Ausubel *et al.* (1989). Effectively, DNA was transferred by capillary action from the gel to nitrocellulose in 5 × SSC (75 mM trisodium citrate, pH 7.0, 750 mM NaCl). DNA was completely transferred in 3 to 12 h. After transfer was complete, the nitrocellulose was washed briefly in 5 × SSC to remove any adhering gel pieces, and the DNA was fixed to the nitrocellulose by ultraviolet light cross-linking at 1200 J/cm ^{2} using a Stratalinker 1800 (Stratagene, La Jolla, CA). Blots were then pre-hybridized in 1.25 × SSC, 0.16 × Denhardt's solution (0.0016% Ficoll, 0.0016% polyvinylpyrolidone, 0.0016% bovine serum albumin), 4 µg sheared salmon sperm DNA/ml, 0.01% SDS, 20 mM sodium phosphate, pH 7.0, for 3 h at 65°C. Hybridization was carried out in the same buffer containing 30% deionized formamide and 0.5-1 × 10^6 cpm/ml of radiolabelled probe or 10 ng of HRP-conjugated probe/ml and incubated for more than 12 h at 42 °C. Nitrocellulose blots were washed four times for 15 min each in 1 × SSC, 0.1% SDS at 55°C , followed by exposure to film.

2.2.7 *DNA sequencing*

Sequencing of the *PEX23* gene was performed by the method of Sanger *et al.* (1977) using Sequenase (USB). Reactions were resolved on 5% Long Ranger acrylamide

gels (J.T. Baker) containing 8 M urea and 1.2 × TBE. Gels were run in 0.6 × TBE at 60 watts/gel. Gels were dried and exposed to Kodak X-Omat film for 1 to 3 d.

Additional sequencing was performed with an ABI Prism 310 automated DNA sequencer using BigDye Terminator Cycle Sequencing (Applied Biosystems).

2.2.8 Oligonucleotide primers

All oligonucleotide primers were synthesized on a Beckman Oligo 1000M DNA Synthesizer. Key oligonucleotides are listed in Table 2.1.

2.3.0 Analytical procedures

2.3.1 Glass bead lysates

Total cellular protein was isolated by glass bead disruption of cells in 25 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM EDTA, 10% (v/v) glycerol, 100 mM DTT containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM NaF, and pepstatin, antipain, leupeptin and aprotinin each at 1 μg/ml). Cell suspensions were vortexed four times for 1 min each and placed on ice for 1 min between each vortex. The suspensions were subjected to centrifugation in a microfuge for 5 min to pellet unbroken cells and glass beads. Supernatants were removed and stored at -20°C.

2.3.2 Protein determination

Total protein was measured according to Bradford (1976) using a protein assay kit (BioRad) and bovine serum albumin as the standard. Samples were mixed with 1 ml of

dye reagent, vortexed briefly and incubated for 5 min at room temperature. Samples were quantitated by absorbance at 595 nm with a Beckman DU-60 spectrophotometer.

2.3.3 Electrophoresis and protein blotting

Protein samples were resolved according to the method of Laemmli (1970). Samples were denatured by boiling 5 min in SDS-PAGE sample buffer and then separated by discontinuous SDS-PAGE on a 10% gel. Samples were run at 50 to 200 V in 50 mM Tris-HCl, pH 8.8, 0.4 M glycine, 0.1% SDS until the dye front reached the bottom of the gel. Proteins were directly visualized by staining in 0.1% Coomassie Brilliant Blue (R-250), 10% (v/v) acetic acid, 35% (v/v) methanol. Otherwise, proteins were electrophoretically transferred to nitrocellulose with either a BioRad western transblotter apparatus overnight at 100 mA in 20 mM Tris-HCl, pH 7.5, 150 mM glycine, 20% (v/v) methanol or by semi-dry electrophoretic transfer (Tyler Research Instruments, Edmonton, AB) for 1.5 h at 0.75 mA/cm^2 of transfer area, as described (Kyshe-Anderson, 1984).

2.3.4 Immunoblot analysis

Protein blots were blocked in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl , 0.05% (w/v) Tween 20) containing 5% skim milk powder for 1 h. Blocking solution was removed and replaced with a primary antibody solution in TBST containing 1% skim milk powder. Incubation with primary antibody was for 1 h at room temperature or overnight at 4°C with agitation. Blots were washed three times for 10 min each with TBST and then incubated at room temperature for 1 h with secondary detection

antibodies diluted in TBST containing 1% skim milk powder. Blots were again washed three times for 10 min each in TBST, processed for detection by enhanced chemiluminescence and exposed to Kodak X-Omat XK-1 film.

2.3.5 Enzymatic assays

i) Catalase

Catalase activity was measured by combining 50 mM potassium phosphate buffer (pH 7.5) with 1 to 50 µl of sample to a final volume of 950 µl in a quartz cuvette. 50 µl of 0.3% H_2O_2 was added, and the sample was set to zero absorbance. The OD_{240} was measured at 10 s intervals for 2 min to monitor the consumption of H_2O_2 in the reaction. The OD_{240} decreased in the presence of catalase activity (ücк, 1963).

ii) Cytochrome c oxidase

Cytochrome *c* oxidase activity was measured by combining 1 to 20 µl of sample in 50 mM potassium phosphate buffer (pH 7.0) and 0.38 mg reduced horse heart cytochrome c/ml. Cytochrome c was reduced by the addition of a minute amount of sodium hydrosulfite. Excess sodium hydrosulfite was removed by size exclusion filtration on Sephadex G50 equilibrated with 0.03 M ammonium acetate buffer (pH 7.5). Oxidation of cytochrome c was monitored as a decrease in OD_{550} at 5 s intervals over 2 min (Douma *et al.*, 1985).

2.4.0 Yeast cultivation and analysis of the strain *pex23-1*

2.4.1 Strains and culture conditions

The *Y. lipolytica* strains used in this study are listed in Table 2.2. Growth was at 30°C. Strains containing plasmids were grown in minimal media (YND, YNA or YNO). Strains not containing plasmids were grown in rich media (YEPD, YEPA or YPBO). Media components were as follows: YND, 1.34% yeast nitrogen base without amino acids, Complete Supplement Mixture minus the appropriate amino acids (Bio101, La Jolla, CA) at twice the manufacturer's recommended concentration (2 × CSM), 2% glucose; YNA, 1.34% yeast nitrogen base without amino acids, 2 × CSM, 2% acetate; YNO, 1.34% yeast nitrogen base without amino acids, 2 × CSM, 0.05% Tween 40 (w/v), 0.1% oleic acid (w/v); YEPD, 1% yeast extract, 2% peptone, 2% glucose; YEPA, 1% yeast extract, 2% peptone, 2% acetate; YPBO, 0.3% yeast extract, 0.5% peptone, 0.5% K_2HPO_4, 0.5% KH_2PO_4, 1% Brij-35, 1% (w/v) oleic acid. For the induction of peroxisomes, strains were grown overnight in glucose-containing medium to an OD_{600} of 1 to 2, harvested by centrifugation, diluted 1:4 in oleic acid-containing medium and then grown for an additional 8 h.

2.4.2 *Mutagenesis of Y. lipolytica and negative selection of mutants*

The *pex23-1* mutant strain was isolated from randomly mutagenized *Y. lipolytica* strain *E122* as follows. *E122* cells were grown overnight at 30°C in YEPD to an OD_{600} of 1. Five ml of culture was subjected to centrifugation in a clinical centrifuge and washed twice with one ml sterile water and one ml sodium phosphate buffer (pH 7.0). Cells were resuspended in one ml sodium phosphate buffer (pH 7.0), and 50 µl of ethy methanesulfonate (EMS) was added, followed by incubation at room temperature. At 15 min intervals, 0.2 ml of cell suspension was removed and added to eight ml of sodium

thiosulfate to inactivate the EMS. Cells were harvested, washed three times with water and resuspended in YEPD. A cell death rate of 60% to 80% was selected in order to ensure that all genes of *Y. lipolytica* were subjected to mutation. Mutated cells were plated onto YEPD agar plates, grown for 2 d at 30 °C and then replica plated onto YNA and YNO agar plates. Colonies that grew on YEPD and YNA plates but not YNO plates were selected for further analyses.

2.4.3 Transformation of Y. lipolytica

50 ml of a YEPA culture of *Y. lipolytica* was grown at 30 °C overnight to an OD_{600} of 0.5 to 1.0. Cells were harvested by centrifugation, resuspended in 50 ml of TE7.5 (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 100 mM lithium acetate and incubated at 30°C for 30 min with shaking at 100 rpm. 1 M DTT was added to a final concentration of 20 mM to the cells and incubated for a further 15 min at 30 °C with shaking at 100 rpm. Cells were harvested by centrifugation and washed once with water at room temperature, once with water at 4°C and once with 1 M sorbitol at 4 °C. Harvested cells were resuspended in the residual sorbitol and placed on ice. 20 µl aliquots of the cell suspension were combined with 1 to 3 µl of DNA solution, suspended between the bosses of a chilled Gibco electroporation chamber and subjected to electroporation in a Gibco Cell-Porator at 250 V and 16 kΩ resistance. Actual voltage across the sample was approximately 1.55 kV. Cells were immediately transferred to 100 µl of sorbitol and spread on YNA-agar selective plates. Colonies were observed after 3 d of growth at 30°C. Transformation efficiency was sometimes increased by plating cells

on YNA-agar plates containing 1 M sorbitol; however, colonies took 1 to 2 d longer to observe.

2.4.4 Isolation of the PEX23 gene

The *PEX23* gene was isolated by functional complementation of the *pex23-1* strain with a *Y. lipolytica* genomic DNA library in the autonomously replicating *E. coli* shuttle vector pINA445 (Nuttley *et al*., 1993) (Section 2.1.4). Leu$^+$ transformants were replica plated onto selective YNO agar plates and screened for their ability to utilize oleic acid as a sole carbon source. Total DNA was isolated from colonies that recovered growth on YNO and introduced by electroporation into *E. coli* for plasmid recovery. Restriction fragments prepared from the genomic insert were subcloned and tested for their ability to functionally complement the *pex23-1* strain (performed by Dr. Melchior Evers). The smallest genomic DNA fragment capable of complementation was sequenced in both directions (performed by Dr. Richard Rachubinski).

2.4.5 Integrative deletion of the PEX23 gene

Targeted integrative deletion of the *PEX23* gene was performed with the *URA3* gene of *Y. lipolytica*. A 1.7-kbp *Sal*I fragment containing the *URA3* gene was inserted into a plasmid containing the *PEX23* gene locus cut with *Eco*RV and *Stu*I, thereby replacing a 2.2-kbp fragment containing the entire *PEX23* ORF with the *URA3* gene. This construct was then cleaved with *Bam*HI and *Xba*I to liberate the *URA3* gene flanked by 1068 bp and 1407 bp of the 5' and 3' regions, respectively, of the *PEX23* gene. The resultant linear construct was used to transform *Y. lipolytica* strains *E122* and *22301-3* to

uracil prototrophy. Ura^+ transformants were selected and screened for their inability to grow on YNO agar. Correct integration of the *URA3* gene at the *PEX23* gene locus was confirmed by Southern blot analysis. Deletion strains were crossed with wild-type strains and the *pex23-1* mutant strain, and the resultant diploids were checked for growth on YNO agar.

2.4.6 *Mating and genetic analysis*

Mutants were characterized by standard genetic techniques for *Y. lipolytica* (Gaillardin *et al.*, 1973). Diploid strains were generated by growing haploid strains on PSM-agar (1% yeast extract, 0.2% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 2% glucose, 2% agar) overnight at 30°C (Table 2.2). Haploid strains of each mating type were mixed and grown on YM-agar (0.3% yeast extract, 0.5% peptone, 0.3% malt extract, 0.5% glucose, 2% agar) for 4 d at 30 °C. After 4 d, mating yeast from YM-agar were streaked on selective YND plates (supplemented for haploid auxotrophic requirements) to select for diploids. These diploids were isolated and tested for growth on oleic acid-containing medium.

2.4.7 *Epitope-tagging of Pex23p*

The Pex23p protein was tagged at its C-terminus with three tandem copies of the human c-Myc epitope consisting of the amino acid sequence "EQKLISEEDL" (Kolodziej and Young, 1991). The open reading frame (ORF) and termination codon of the *PEX23* gene, along with approximately 1.6 kbp of genomic DNA 5' to the ORF, were amplified by PCR using primers 706 (5') and 805 (3') (Table 2.1). The amplified product was

digested with *Bam*HI and *Sal*I and inserted into the plasmid pSP73 (Promega) cut with the same enzymes to yield plasmid pPEX23-5. Approximately 1.8 kbp of the 3' flanking region of the *PEX23* gene was also amplified by PCR using primers 806 (5') and 807 (3') (Table 2.1). This PCR product was digested with *Xho*I and *Xba*I and inserted into the same sites of pGEM7Zf(+) to yield plasmid pPEX23-3. The insert of pPEX23-5 was liberated by cleavage with *Bam*HI and *Xho*I and ligated into the same sites of pPEX23-3 to make pPEX23-53. Next, a DNA fragment coding for three tandem copies of the c-Myc epitope was excised from the plasmid pCR2.1 (a kind gift of Dr. David Stuart, University of Alberta) with *Sal*I and *Xho*I and inserted in-frame and downstream of the *PEX23* gene ORF in pPEX23-53 to make the plasmid pPEX23-Myc expressing the chimeric protein Pex23p-Myc. pPEX23-Myc was tested for its ability to restore growth on oleic acid and peroxisome assembly to the *pex23-1* and *pex23KOA* mutant strains. Pex23p-Myc was detected immunologically with mouse monoclonal antibody 9E10.

2.4.8 Construction of pPEX23-TH

Plasmid pTC3, containing the promoter and terminator regions of the *Y. lipolytica* thiolase gene separated by a unique *Eco*RI site, was previously constructed by Ms. Jennifer Smith, University of Alberta, as follows. The promoter and terminator regions of the thiolase gene were amplified by PCR from the plasmid pS106 (Berninger *et al.*, 1993) using the oligonucleotide primers THpr5'/THpr3' and THtr5'/THtr3', respectively (Table 2.1). The amplified products were cloned into pGEM7Zf(+) to make the expression cassette vector pTEC with a unique *Eco*RI cloning site between the promoter

and the terminator regions. The cassette was then cloned into the *Bgl*II site of a modified pINA443 plasmid with its *Eco*RI site deleted to make the plasmid pTC3.

The ORF of *PEX23* was amplified by PCR from the plasmid p23H2 using the primers 815 and 816 to engineer *Eco*RI sites at the ends of the *PEX23* ORF. This construct was cloned into the *Eco*RI site of pTC3 to make the overexpression plasmid, pPEX23-TH. This plasmid was introduced by transformation into wild-type *Y. lipolytica E122* to make the strain *P23TH* (Table 2.2).

2.5.0 Microscopy

2.5.1 Electron microscopy

Electron microscopy was performed essentially as described (Goodman *et al.*, 1990). *Y. lipolytica* strains were grown in rich glucose-containing medium overnight or in either rich or selective oleic acid-containing media for 8 h. Cells were fixed for 20 min at room temperature in 1.5% $KMnO_4$, washed three times in water and immersed in 2% uranyl acetate for 1 h at room temperature or overnight at 4°C. Cells were then dehydrated by immersion in a series of ethanol washes (60%, 80%, 95%, 100%) for 10 min each and in three changes of propylene oxide of 10 min each. Cells were infiltrated with propylene oxide/TAAB 812 resin (1:1) for 1 h at room temperature, embedded in fresh TAAB 812 resin and cured for 48 h at 60 °C. Resin blocks were sectioned, and sections were observed with a Phillips 410 electron microscope.

2.5.2 Immunofluorescence microscopy

Immunofluorescence microscopy of yeast cells was performed essentially as described (Pringle *et al.*, 1991). Cell cultures were grown overnight (or for 8 h if grown in oleic acid-containing medium) in 10 ml of medium. 1 ml of 37% formaldehyde was added directly to 9 ml of culture, and the suspension was incubated for 30 to 60 min at room temperature to fix the cells. The cells were harvested with a clinical centrifuge and washed with 4 ml of Solution B (100 mM potassium phosphate, pH 7.5, 1.2 M sorbitol). Cells were converted to spheroplasts in Solution B (~100 µl of cells/ml Solution B) containing 28 mM 2-mercaptoethanol and 40 µl (2 mg/ml) of Zymolyase 100T for 30 to 60 min at 30°C in an incubator wheel rotating at 100 rpm. Spheroplasts were adhered to glass slides (Marienfeld, Germany) pre-treated with poly-L-lysine and were permeabilized by immersion for 6 min in methanol and 30 s in acetone at -20 °C. Slides were incubated with primary antibodies made up in TBST and 1% milk for 30 to 60 min. Slides were then incubated with FITC (fluorescein)- or TRITC (rhodamine)-conjugated secondary antibodies (Section 2.1.6, ii) in TBST and 1% milk for 30 to 60 min. Slides were washed 10 times with TBST after each incubation. Slides were viewed on an Olympus BX50 fluorescence microscope, and photographs were taken with a Spot Cam digital fluorescence camera.

2.6.0 Cell fractionation and peroxisome isolation

2.6.1 Subcellular fractionation

Fractionation of oleic acid-grown cells was performed as previously described (Szilard *et al.* , 1995) and included the differential centrifugation of lysed and homogenized spheroplasts at 1,000 × g_{max} for 8 min at 4°C in a Beckman JS13.1 rotor to

yield a postnuclear supernatant (PNS) fraction. The PNS fraction was further subjected to differential centrifugation at 20,000 × g_{max} for 30 min at 4°C in a JS13.1 rotor to yield a pellet (20KgP) fraction enriched for peroxisomes and mitochondria and a supernatant (20KgS) fraction enriched for cytosol. Peroxisomes were purified from the 20KgP by isopycnic centrifugation on a discontinuous sucrose (25, 35, 42, and 53% w/w) gradient in a Beckman VTi50 rotor at 84,500 × g_{av} for 1 h at 4°C (performed by Dr. Vladimir Titorenko). Fraction 4, equilibrating at a density of 1.21 g/cm ^{3} and containing the peak activity for the peroxisomal marker protein, catalase, was recovered. The peroxisomal fraction was further purified as described (Titorenko *et al.*, 1996). Four volumes of 0.5 M sucrose in Buffer H (5 mM MES, pH 5.5, 1 mM KCl, 0.5 mM EDTA, 0.1% (v/v) ethanol) was added to fraction 4. Peroxisomes were sedimented through a 150- µl cushion of 2 M sucrose in buffer H by centrifugation at 200,000 × g_{max} for 20 min at 4°C in a Beckman TLA120.2 rotor. The resultant pellet was resuspended in buffer H containing 1 M sorbitol and was subjected to further centrifugation on a linear 20-60% (w/w) sucrose gradient (in buffer H) at 197,000 × g_{av} for 18 h at 4°C in a Beckman SW41Ti rotor. The peak peroxisomal fraction, fraction 5, equilibrating at a density of 1.21 g/cm^3 was recovered.

2.6.2 *Flotation gradient analysis*

The 20KgP fraction from the *pex23KOA* mutant strain was subjected to a two-step flotation gradient analysis to detect the presence of vesicular structures containing peroxisomal proteins. The 20KgP was resuspended in 100 µl of 30% (w/w) sucrose and 0,5 M sorbitol in buffer H and mixed with 300 µl of 65% (w/w) sucrose in buffer H. The

sample was transferred to a 5-ml centrifuge tube and overlaid with 2.3 ml of 50% (w/w) sucrose and 2.3 ml of 20% (w/w) sucrose (both in buffer H). Gradients were subjected to centrifugation in a Beckman SW50.1 rotor at 200,000 × g_{av} for 18 h at 4°C. Gradients were fractionated from the top, and 18 fractions of ~275 µl each were collected.

The 20KgS fraction from the *pex23KOA* mutant strain was subjected to centrifugation at 200,000 × g_{max} for 30 min at 4°C to yield a pellet (200KgP) fraction and a supernatant (200KgS) fraction consisting essentially of cytosol. The 200KgS fraction was divided into two equal aliquots. The first aliquot was incubated for 2 h at 75 °C. Under these conditions, all cytosolic proteins formed insoluble aggregates, as judged by light scattering at 320 nm and as confirmed by SDS-PAGE followed by Coomassie staining. Aggregates of cytosolic proteins were pelleted by centrifugation at 20,000 × g_{max} for 30 min at 4°C, resuspended in 100 µl of 30% (w/w) sucrose and 0.5 M sorbitol in buffer H, and mixed with 300 µl of 65% (w/w) sucrose in buffer H. This material was subjected to flotation on a two-step sucrose gradient as described above. The second aliquot of the 200KgS fraction (in buffer H supplemented with 1 M sorbitol) was concentrated to final volume of 50 µl by centrifugation through a Biomax-30 filter (Millipore) at 7,200 × g_{max} for 40 min at 4°C. The concentrated soluble proteins were mixed with 50 µl of 60% (w/w)sucrose and then with 300 µl of 65% (w/w) sucrose (both in buffer H), and subjected to flotation on a two-step sucrose gradient as described above. Gradient fractions were assayed for protein and sucrose density and for the presence of peroxisomal proteins (performed by Dr. Vladimir Titorenko).

2.6.3 Sodium carbonate extraction of peroxisomal membranes

Peroxisome subfractions were prepared from purified peroxisomes essentially as described (Eitzen *et al.*, 1997). 150 µg of purified peroxisomes were lysed by the addition of 10 volumes of ice-cold Ti8 buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM PMSF, 5 mM NaF, and pepstatin, leupeptin and aprotinin each at 1 µg/ml) and subjected to centrifugation at 100,000 $\times g_{max}$ for 30 min at 4°C. Half of the resultant pellet was then treated with 0.1 M Na_2CO_3 (pH 11.5), followed by centrifugation as above. Proteins were precipitated by addition of TCA to 10% and washed with 80% (v/v) acetone (performed by Dr. Vladimir Titorenko).

2.6.4 Protease protection

For protease protection experiments, peroxisomes purified by isopycnic centrifugation were diluted with 4 volumes of 0.5 M sucrose in buffer H. Peroxisomes were sedimented onto a 150-µl cushion of 2 M sucrose in buffer H by centrifugation at 200,000 x g_{max} for 20 min at 4°C in a TLA120.2 rotor. The sedimented peroxisomes were resuspended in 850 µl of buffer H containing 1 M sorbitol. Aliquots of 100 µg of protein were incubated with 0, 100, 200 and 500 µg of trypsin for 1 h on ice, in either the presence or the absence of Triton X-100 at 0.5% (v/v) final concentration. The reaction was terminated by addition of TCA to a final concentration of 10%, and the protein precipitates were washed with acetone as above. Equivalent fractions from each reaction were subjected to SDS-PAGE, followed by immunoblotting (performed by Dr. Vladimir Titorenko).

Table 2.1. Oligonucleotides

OligoID	5' -----> 3' Sequence	Function
588	CATGGGCTTGAGCTTG	*PEX23* sequencing
589	GCTGCAGGTACTCCTG	
590	TGTATCGGGCAGCTTG	
591	TGGCCTACGAGCGAAC	
600	GCCTCTGGAGAACTTC	
601	TCACTAGCTGTTCCAC	
602	GTTACTGTGTGTGTGC	
603	GTACTGTATACAATTA	
604	TCTAATGCCCTTCGTC	
605	GACGCTGCTGGAGATG	
606	CGGTTCCGAATTTCTC	
615	ACACGATTACAGCGGC	
616	AGCCAGTGCCGCATAG	
617	CAGCACCTAGTTTCAC	
618	TCGTCGGGTGTACTTG	
625	CGGCTCTAAGAAGATC	
626	TACTGGGAAGATTATG	
627	ATCAGTGGGCTTGGTG	
628	GCAAGGTGAAGCCTAC	
647	GACGCCGGTAGTGAGC	
648	GACCACGGTCACGTAG	
649	CTACGTGACCGTGGTC	
651	CGTAGTGTGCTTGGTC	
652	GTGAGCATCATGCAAG	
653	GATCTGATGCTGAATG	
706	TTCACACAGGAAACAGCTATGACCATG	Pex23p-My construction
805	CTCGAGGACTCCGTCGACTCTCTTAGAGT CCTCCTCGAATCTAATGC	
806	GTCGACGGAGTCCTCGAGTAATTAAATA TATGAATGTATCAT	
807	ATTCTAGATCGACTGGTCCAAAGTTGTGG	
THpr5'	CAGATCTAACCTACCGG	*PEX23* in thiolase overexpression construct
THpr3'	TGAATTCGGTCCAAAGTG	
THtr5	GAGTGAATTCACATACAA	
THtr3	GAGATCTACGACCTGG	
815	TAGAATTCATGTCGGATAAGGAGAAGAA AAAGAGC	
816	TAGAATTCTTATCTCTTAGAGTCCTCCTC GAATC	

2.2. *Y. lipolytica* strains used in this study

Strain[a]	Genotype
E122	*MATA, ura3-302, leu2-270, lys 8-11*
22301-3	*MATB, ura3-302, leu2-270, his1*
pex23-1	*MATA, ura3-302, leu2-270, lys 8-11, pex23-1*
P23TR	*MATA, ura3-302, leu2-270, lys8-11, p23E4(LEU2)*
pex23KOA	*MATA, ura3-302, leu2-270, lys8-11, pex23::URA3*
pex23KOB	*MATB, ura3-302, leu2-270, his1, pex23::URA3*
D1-23	*MATA/MATB, ura3-302/ura3-302, leu2-270/leu2-270, lys8-11/+, +/his1, pex23-1/+*
D2-23	*MATA/MATB, ura3-302/ura3-302, leu2-270/leu2-270, lys8-11/+, +/his1, pex23::URA3/+*
D3-23	*MATA/MATB, ura3-302/ura3-302, leu2-270/leu2-270, lys8-11/+, +/his1, + /pex23::URA3*
D4-23	*MATA/MATB, ura3-302/ura3-302, leu2-270/leu2-270, lys8-11/+, +/his1, pex23-1/pex23::URA3*
P23-Myc	*MATA, ura3-302, leu2-270, lys8-11, pPEX23-Myc(LEU2*
P23-Myc (Int)	*MATA, ura3-302, leu2-270, lys8-11, pex23::URA3::PEX23-Myc*
P23TH	*MATA, ura3-302, leu2-270, lys8-11, pPEX23TH (URA3*

[a]Strains *E122* and *22301-3* were from Dr. Claude Gaillardin, INRA, Thiverval-Grignon, France. All other strains were from this study.

3.0 Results

3.1 Mutagenesis and selection of mutants

In this thesis, a negative selection procedure is used to identify mutants that cannot grow on the fatty acid oleate as a carbon source (ole⁻ phenotype). Since all β-oxidation of fatty acids occurs in the peroxisome in *Y. lipolytica*, this screen identifies strains that may be compromised in the assembly of peroxisomes. To increase the mutation frequency, yeast cells are exposed to a chemical mutagen, ethyl methanesulfonate (EMS).

Growing cultures of *Y. lipolytica E122* were treated with EMS and plated onto YEPD agar plates at a concentration of ~800 colonies/plate. Once large enough, the colonies were replica plated onto YNA (acetate) and YNO (oleate) plates. Colonies that grew on YNA but not YNO plates were selected for further analysis (Figure 3.1). Screening the mutants on acetate medium eliminates the possibility of selecting strains whose apparent inability to utilize oleate as a carbon source derives from a downstream mitochondrial mutation. Such mutants cannot use the acetyl-CoA units provided by the β-oxidation of fatty acids and therefore will not grow on peroxisome-requiring oleate nor on mitochondrial-requiring acetate.

The initial screen revealed seven complete or partial ole⁻ strains. Some strains grew slowly on oleate as compared to the wild-type strain, and some strains did not grow at all. Some strains were found to be either cold-sensitive or temperature-sensitive mutants by monitoring growth at 16°C and 33°C. The results of the screen are summarized in Table 3.1. The two mutants displaying the strongest ole⁻ phenotype,

named *mut 25* and *mut 28* (hereafter referred to as *pex 23-1*) were selected for further morphological and biochemical analyses.

Table 3.1. Ole⁻ mutants identified by the mutagenesis screen

Mutant	Growth on Oleate			Growth on Acetate		
	16°C	30°C	33°C	16°C	30°C	33°C
mut1	-	-	-/+	+	+	+
mut7	-	-/+	+	+	+	+
mut17	-	+	+	+	+	+
mut18	-	-	-	+	+	+
mut 25	-	-	-	+	+	+
mut28/pex23-1	-	-	-	+	+	+
mut33	+	-	-	+	+	+

Legend: - , no growth; -/+, partial or slow growth; +, wild-type growth

3.2 Identification and characterization of the *PEX23* gene

Both mutant strains were transformed with a *Y. lipolytica* genomic DNA library to try to functionally complement the ole$^-$ phenotype. Unfortunately, *mut25* was unable to be functionally complemented by the library after screening approximately 5×10^6 leucine prototrophy transformants.

The gene that functionally complemented the *pex23-1* strain was isolated from the library after screening approximately 2×10^5 leucine prototrophy transformants and identifying one strain that had recovered growth on oleic acid (ole$^+$ phenotype) (Figure 3.2). Total DNA was isolated from this strain and the complementing plasmid was recovered by transformation of *E. coli*. The plasmid insert was mapped by restriction endonuclease digestion, and fragments of the insert resulting from the various digestions were cloned and introduced by transformation into the *pex23-1* strain to delineate the region of complementation (Figure 3.3). This region localized to a unique *Nco*I site within the initial complementing insert. DNA sequencing revealed an ORF of 1254 nucleotides coding for a protein of 418 amino acids, Pex23p, having a predicted molecular weight of 47,558 (Figure 3.4). Pex23p does not contain PTS1 or PTS2 motifs, and although a C-terminal PTS1-like tripeptide SKR is present, it is not necessary for function (see below). A search of protein databases using the GENINFO(R) BLAST Network Service (Blaster) of the National Center for Biotechnology Information revealed three homologous proteins coded for by the ORFs YLR324W, YGR004W, and

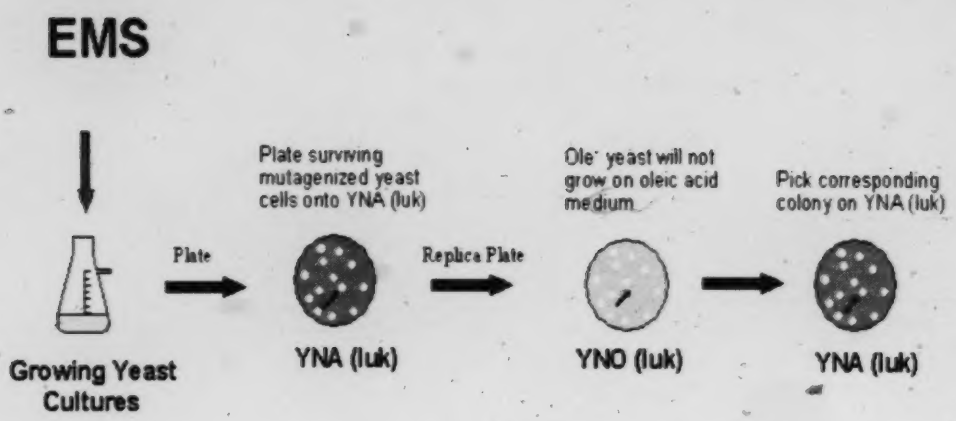

Figure 3.1. Schematic representing the mutagenesis screen and the negative selection procedure used to isolate mutants unable to grow on oleic acid-containing medium. EMS = ethyl methanesulfonate, a chemical mutagen. YNA = agar plates containing acetate as the carbon source. YNO = agar plates containing oleic acid as the carbon source.

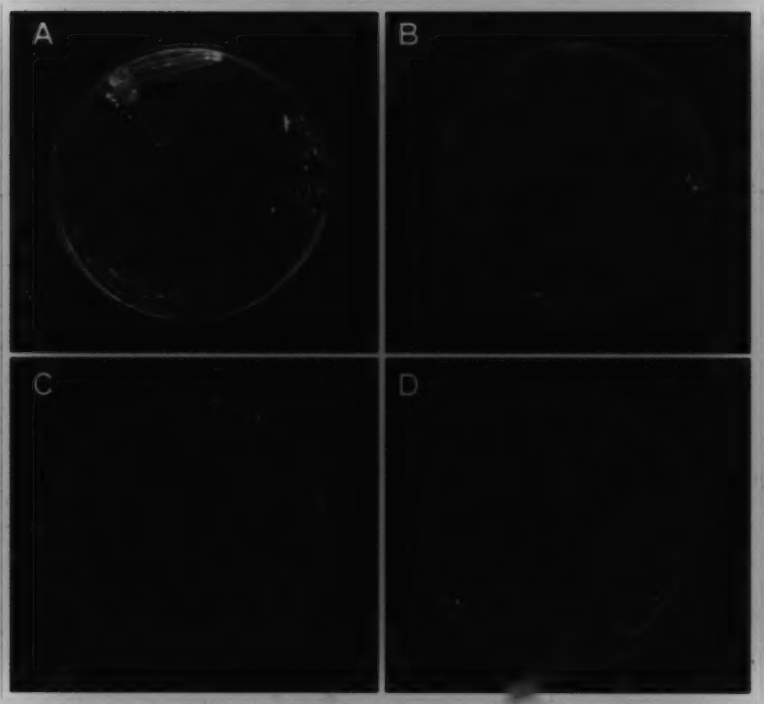

Figure 3.2. Growth of various *Y. lipolytica* strains on oleic acid-containing medium. Appearance of the original mutant strain *pex23-1* and the disruptant strain *pex23KOA* in comparison to the wild-type strain, *E122* (A), and to the complemented strain, *P23TR* (B). Diploid strains *D1-23, D2-23, D3-23,* and *D4-23* (C and D) demonstrate the recessive nature of the *pex23* mutation and that the mutations are allelic. *P23-Myc* (B) and *P23Myc (Int)* (C) are the plasmid-carrying and integrated strains, respectively, for Pex23p-Myc expression. *22301-3*, a second wild-type strain, was not supplemented for its auxotrophic requirements.

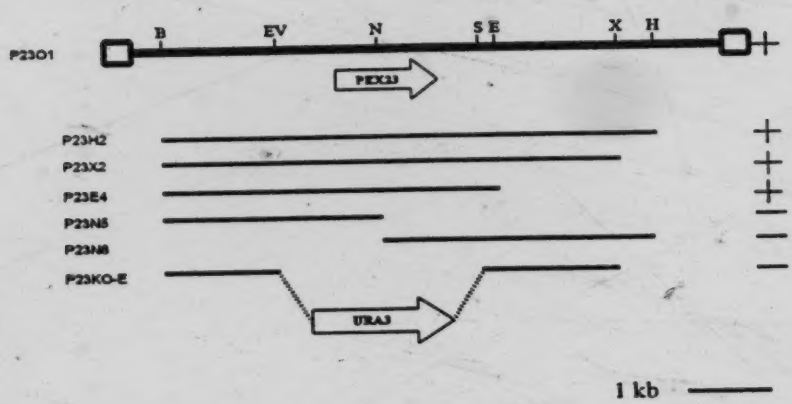

Figure 3.3. Cloning of the *PEX23* gene and *PEX23* deletion strategy. Complementing activity of inserts, restriction map analysis, and targeted gene deletion strategy for the *PEX23* gene. The original complementing insert DNA in the plasmid p23O1 is denoted by the thick black line. Solid lines, *Y. lipolytica* genomic DNA. Boxes, vector DNA. The ORFs of the *PEX23* and *URA3* genes and their directionality are indicated by the wide arrows. The (+) symbol denotes the ability and the (-) symbol the inability of an insert to confer growth on oleic acid to strain *pex23-1*. B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; N, *Nco*I; S, *Stu*I; X, *Xba*I.

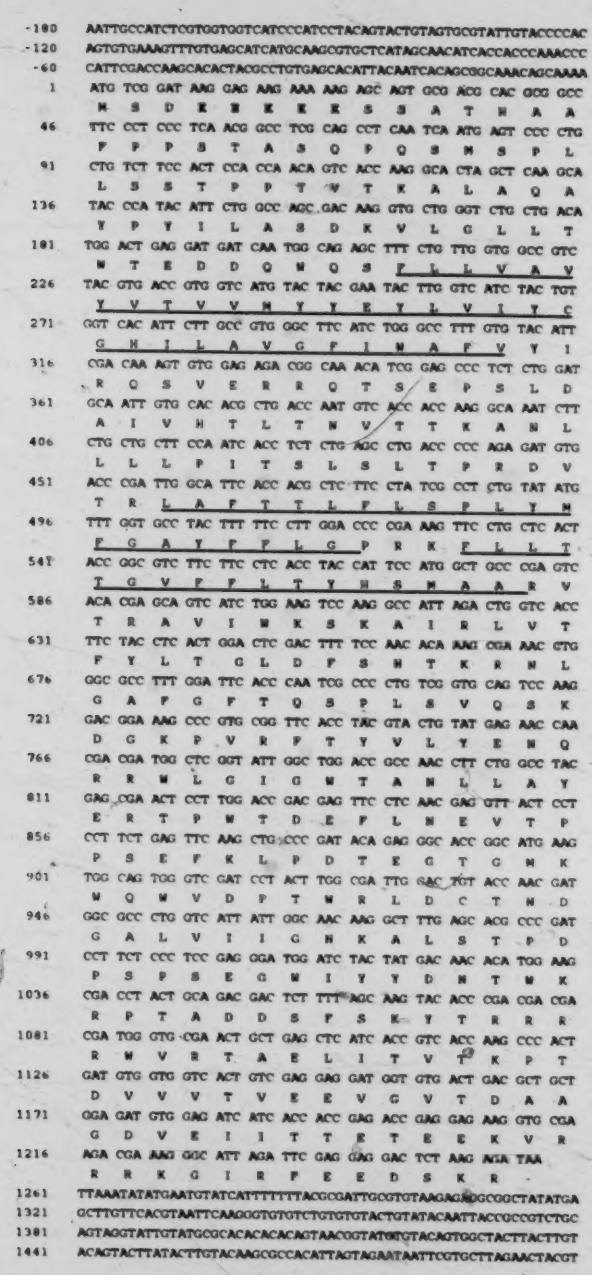

Figure 3.4. Analysis of the *PEX23* gene. Nucleotide sequence of the *PEX23* gene and deduced amino acid sequence of Pex23p. Underlined residues indicate predicted membrane-spanning domains. A potential mPTS is highlighted. These sequence data have been deposited in the DDBJ/EMBL/GenBank databases under accession number AF160511.

YBR168W of the *S. cerevisiae* genome (Figure 3.5). The putative proteins coded for by the ORFs YLR324W and YGR004W are the most similar, having identities and similarities of I = 43%, S = 60.5%, and I = 40%, S = 55% with *Y. lipolytica* Pex23p, respectively. The putative protein coded by the ORF YBR168W is much less similar (I = 14%, S = 19%), yet still has some domains of similarity with the above proteins. Amino acid sequence analysis of Pex23p revealed several potential membrane-spanning domains depending on the method used. The method of Rao and Argos (1986) predicts three membrane-spanning segments, while the methods of Eisenberg *et al.* (1984) and Klein *et al.* (1985) predict two.

The putative *PEX23* gene was deleted by targeted integration of the *Y. lipolytica URA3* gene to make the strains *pex23KOA* and *pex23KOB* in the A (*E122*) and B (*22301-3*) mating types, respectively (Table 2.2). The *PEX23* deletion strains were unable to grow on oleic acid (Figure 3-2A) and possessed the same morphological and protein targeting defects as the original *pex23-1* strain (see below). The diploid strains *D1-23* and *D2-23*, made from the mating of strains *pex23-1* and *pex23KOA* with the wild-type strain *22301-3*, could grow on oleic acid-containing medium, as could the diploid *D3-23*, made from the mating of *pex23KOB* and *E122*, demonstrating the recessive nature of the original *pex23-1* mutation and of the *PEX23* gene deletion. The diploid strain *D4-23*, made by mating the original *pex23-1* strain to strain *pex23KOB* (Table 2.2), was unable to grow on oleic acid-containing medium, demonstrating that the respective genes affected are allelic and that the ability to use oleic acid as the sole carbon source required at least one intact copy of the *PEX23* gene (Figure 3.2, panels C and D).

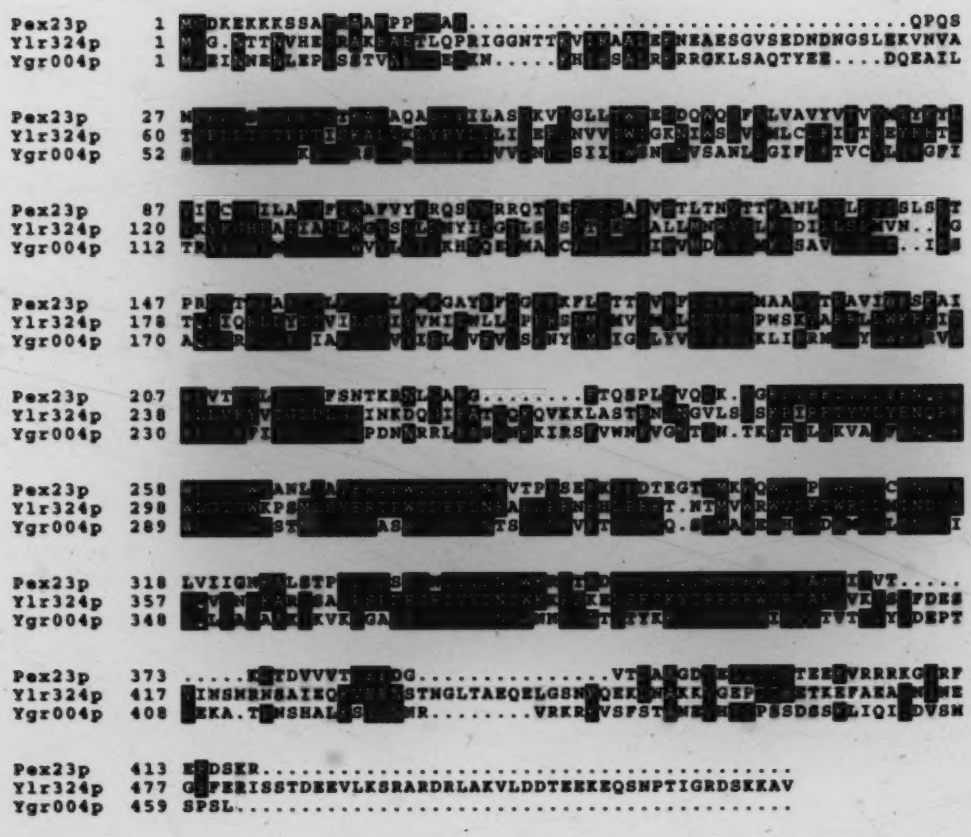

Figure 3.5. Sequence alignment of Pex23p with the hypothetical proteins Ylr324p and Ygr004p encoded by the corresponding ORFs of the *S. cerevisiae* genome. Amino acid sequences were aligned using the ClustalW program. Identical residues (black) and similar residues (gray) in at least half of the proteins are shaded. Similarity rules: G = A = S, A = V, V = I = L = M, I = L = M = F = Y = W, K = R = H, D = E = Q = N, and S = T = Q = N. Dots represent gaps.

3.3 *pex23* cells lack normal peroxisomes but do have vesicular structures containing peroxisomal matrix and membrane proteins

In electron micrographs normal peroxisomes of *Y. lipolytica* appear as round vesicular structures, 0.2 to 0.5 μm in diameter, with a granular electron-dense core and a single unit membrane (Figure 3.6A). The original mutant strain *pex23-1* (Figure 3.6B) and the deletion strain *pex23KOA* (Figure 3.6D) grown in oleic acid-containing medium lacked normal peroxisomes. Both mutant strains accumulated small vesicular structures that were rarely seen in wild-type cells and showed evidence of large membrane sheets surrounding the nucleus. The strain *P23TR* transformed with the *PEX23* gene had the appearance of the wild-type strain and showed normal peroxisome morphology (Figure 3.6C).

Immunofluorescence analysis of oleic acid-grown wild-type cells with anti-SK antibodies and antibodies to the matrix proteins thiolase (THI), isocitrate lyase (ICL) and acyl-CoA oxidase (AOX) showed a punctate pattern of staining characteristic of peroxisomes (Figure 3.7). In contrast, *pex23-1* cells stained with the same antibodies showed a more generalized pattern of fluorescence throughout the cell, characteristic of cytosolic localization (Figure 3.7). The strain *P23TR* transformed with the *PEX23* gene showed characteristic peroxisomal punctate staining with the four different antibodies, while the gene deletion strain *pex23KOA* displayed general cytosolic fluorescence like that of the original *pex23-1* strain (Figure 3.7).

The different strains grown in oleic acid-containing medium were subjected to subcellular fractionation to give a 20,000 x g_{max} pellet (20KgP) enriched for peroxisomes and mitochondria and 20,000 x g_{max} supernatant (20KgS) enriched for cytosol. As

expected, peroxisomal matrix proteins recognized by anti-SKL antibodies and the matrix proteins THI, ICL, and AOX (Figure 3.8A), as well as the classical peroxisomal matrix enzymatic marker catalase (CAT) (Figure 3.8C), were preferentially localized to the 20KgP of wild-type *E122* cells grown in oleic acid-containing medium. The peroxisomal integral membrane peroxin Pex2p and the peripheral membrane peroxin Pex16p were also both preferentially localized to the 20KgP of wild-type cells (Figure 3.8B). In contrast, in the original mutant strain *pex23-1* and in the gene disruption strain *pex23KOA*, all matrix proteins were preferentially mislocalized to the 20KgS (Figure 3.8A and C), although they could also be detected to a much lesser extent in the 20KgP. In contrast, Pex2p and Pex16p were distributed approximately equally between the 20KgP and 20KgS in *pex23* mutant strains (Figure 3.8B). In the wild-type and *pex23* mutant strains, the mitochondrial marker cytochrome *c* oxidase (CCO) was preferentially localized to the 20KgP (Figure 3.8C). Since, in *pex23* mutant strains, all matrix proteins investigated mislocalized preferentially to the 20KgS enriched for cytosol and gave a general fluorescence pattern characteristic of the cytosol, *pex23* mutants are compromised in the import of PTS1 (ICL and anti-SKL proteins) (Barth and Scheuber, 1993), PTS2 (THI), and non-PTS1, PTS2 proteins (AOX) (Wang *et al.*, 1999).

A two-step flotation gradient analysis of the 20KgP fraction from the *pex23KOA* strain was performed to determine whether the peroxisomal matrix and membrane proteins recovered in this fraction were membrane-associated or simply represented large protein aggregates and/or cytosolic contamination of the 20KgP. Flotation of the 20KgP revealed that all peroxisomal proteins floated out of the most dense sucrose and concentrated at the interface between 50% and 20% sucrose (Figure 3.9A and B). In

contrast, both soluble cytosolic proteins and temperature-induced protein aggregates of cytosolic proteins remained at the bottom of the gradient (Figure 3.9B). Therefore, the peroxisomal matrix and membrane proteins recovered in the 20KgP fraction from the *pex23KOA* mutant are present in membrane-associated form, *i.e.* localized to vesicular structures.

3.4 Pex23p-Myc is able to restore biological activity to *pex23-1* and *pex23KOA*

Pex23p was tagged at its C-terminus with the c-Myc epitope (Pex23p-Myc) to permit its detection in cells. The Pex23p-Myc fusion protein migrated to just above the 47.5 kDa molecular weight marker in SDS-PAGE (Figure 3.10). Expression of Pex23p-Myc complemented the *pex23* mutant phenotype and re-established peroxisome formation and the import of peroxisomal matrix proteins, as judged by growth on oleate media (ole^+) (Figure 3.2B and C), immunofluorescence (Figure 3.11A), electron microscopy (Figure 3.11B) and subcellular fractionation (Figure 3.12A). Therefore, Pex23p-Myc mimics the biological activity of wild-type Pex23p.

3.5 Carbonate extraction reveals that Pex23p-Myc is a peroxisomal integral membrane protein

The *pex23KOA* strain expressing Pex23p-Myc (strain *P23-Myc*, Table 2.2) was grown in oleic acid-containing medium and subjected to subcellular fractionation. Pex23p-Myc preferentially fractionated to the 20KgP (Figure 3.12A), as did peroxisomal matrix and membrane proteins (Figure 3.12A and B). Peroxisomes were isolated from the 20KgP fraction by isopycnic centrifugation on a discontinuous sucrose gradient.

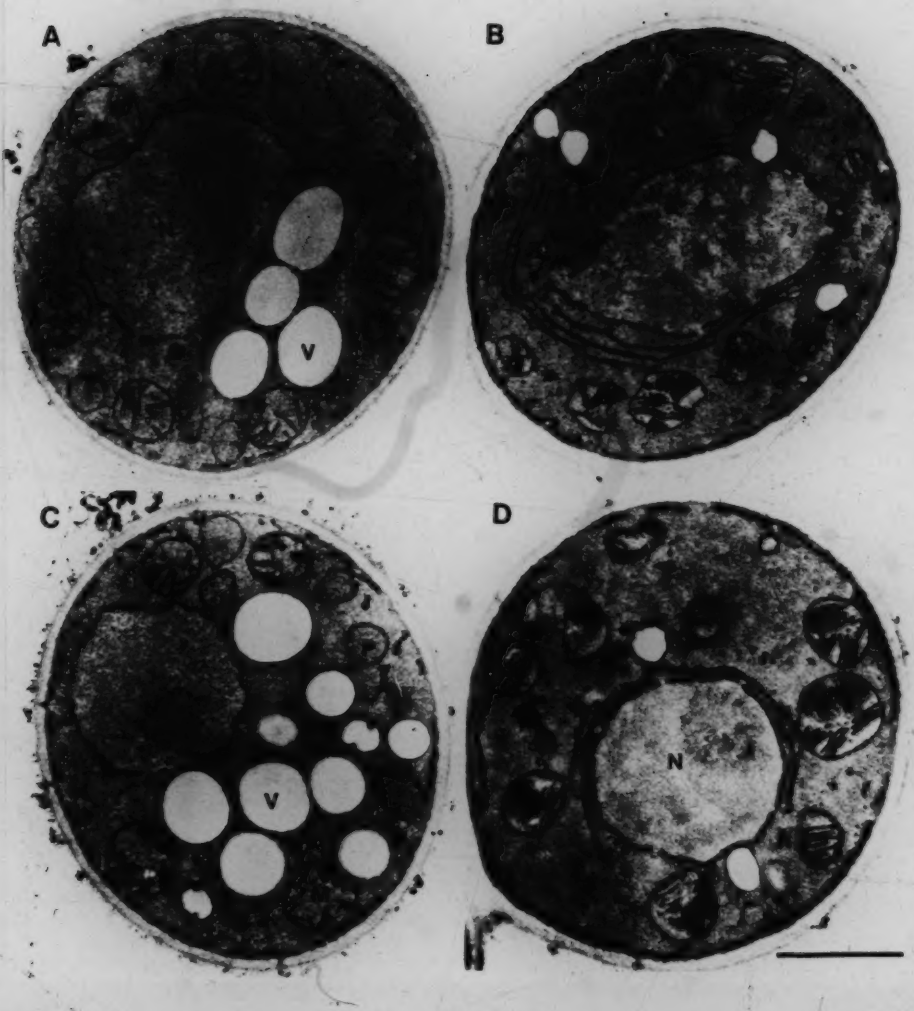

Figure 3.6. Ultrastructure of wild-type, *pex23* mutant, and *PEX23* transformed strains. The *E122* (A), *pex23-1* (B), *P23TR* (C) and *pex23KOA* (D) strains were grown in glucose-containing YEPD medium (YND medium for strain *P23TR*) for 16 h, transferred to oleic acid-containing YPBO medium (YNO medium for strain *P23TR*) and grown for an additional 8 h in oleic acid-containing medium. Cells were fixed in 1.5% $KMnO_4$ and processed for electron microscopy. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar = 1 μm.

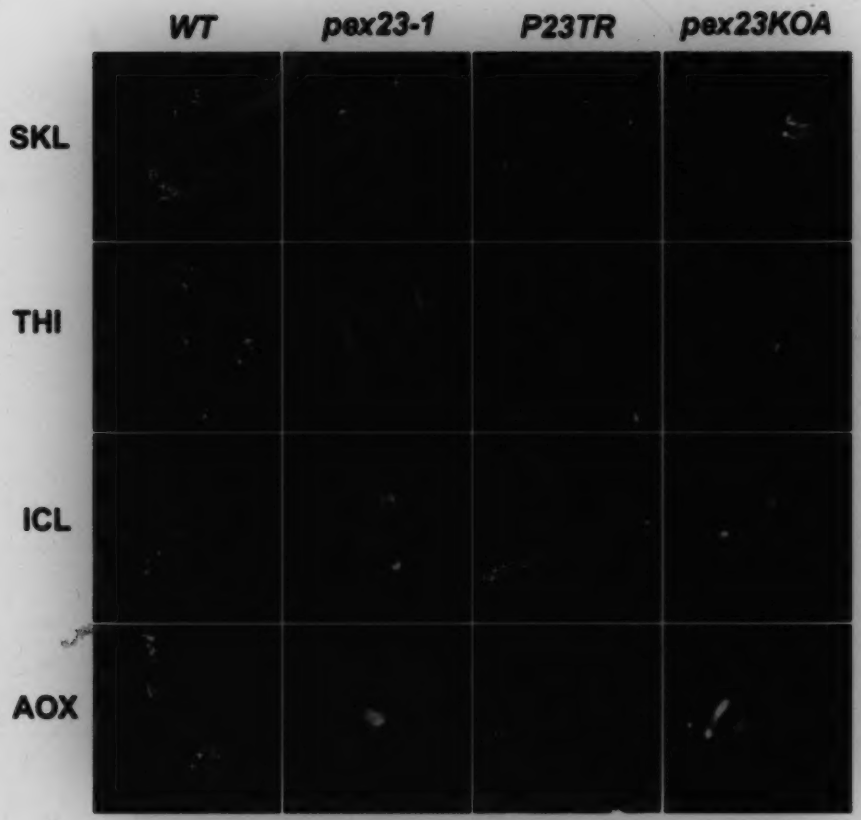

Figure 3.7. Indirect immunofluorescence analysis of wild-type, *pex23* mutant, and *PEX23* transformed strains. Wild-type (*WT*) strain *E122*, mutant strains *pex23-1* and *pex23KOA*, and transformed strain *P23TR* were grown in YPBO medium (YNO medium for strain *P23TR*). Cells were processed for immunofluorescence microscopy with antibodies to the PTS1 tripeptide SKL (SKL), thiolase (THI), isocitrate lyase (ICL) and acyl-CoA oxidase (AOX). Rabbit primary antibodies (SKL, ICL, AOX) were detected with fluorescein-conjugated goat anti-rabbit IgG secondary antibodies, and guinea pig primary antibodies (THI) were detected with rhodamine-conjugated donkey anti-guinea pig IgG secondary antibodies.

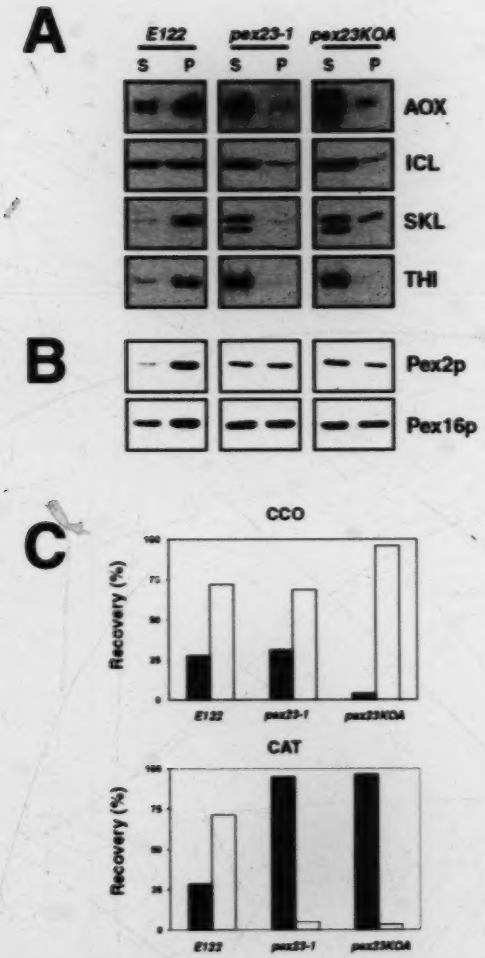

Figure 3.8. Peroxisomal matrix proteins are mislocalized in *pex23* mutant strains. The wild-type strain *E122*, the original mutant strain *pex23-1*, and the gene deletion strain *pex23KOA* were grown in oleic acid-containing YPBO medium and subjected to subcellular fractionation to yield a 20KgP fraction enriched for peroxisomes and mitochondria and a 20KgS fraction enriched for cytosol. (A and B) Equal portions of the 20KgP (P) and 20KgS (S) were analyzed by immunoblotting to the indicated proteins. (C) The activities of catalase (CAT) and cytochrome *c* oxidase (CCO) were assayed enzymatically, and the percentages of enzymatic activity recovered in the 20KgS (closed bars) and the 20KgP (open bars) relative to the total enzymatic activity in the postnuclear supernatant (PNS) fraction are reported.

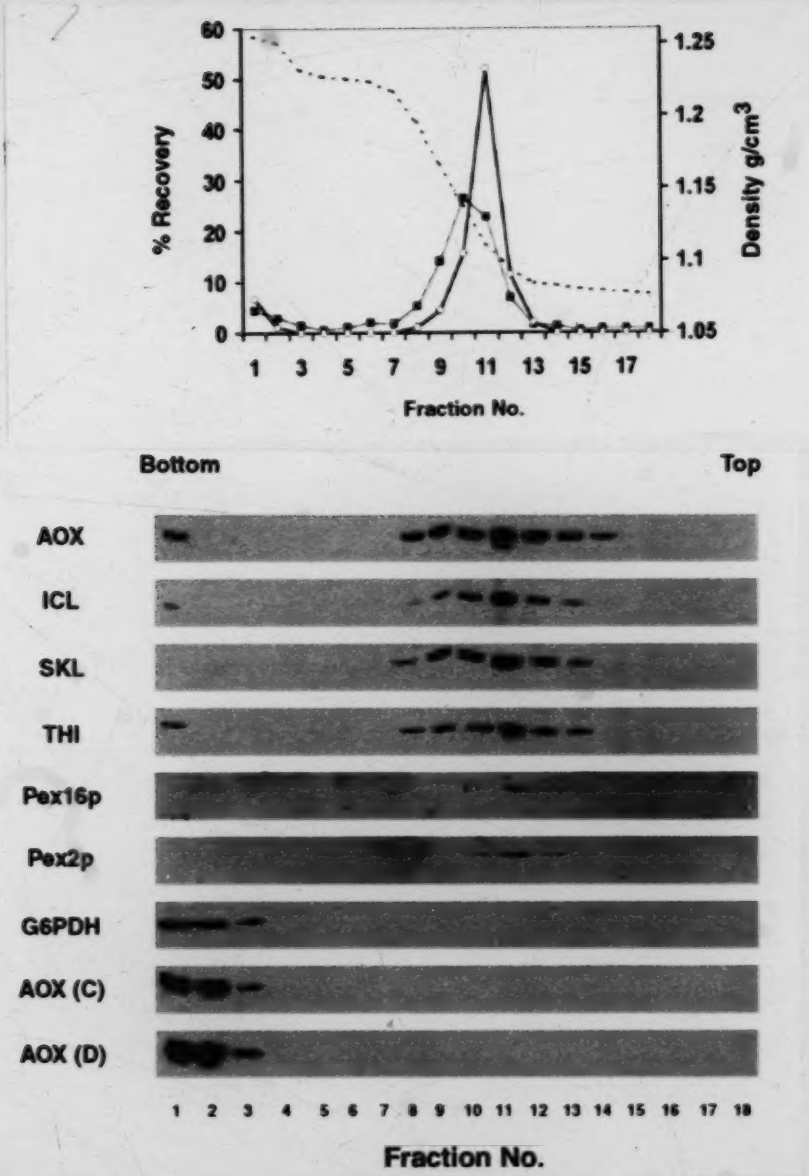

Figure 3.9. Peroxisomal matrix and membrane proteins recovered in the 20KgP fraction of the *pex23KOA* mutant are associated with vesicular structures. The 20KgP fraction, and cytosolic (C) and heat-aggregated (A) proteins from the 200KgS fraction of the *pex23KOA* strain grown in oleic acid-containing YPBO medium were subjected to flotation on a two-step sucrose density gradient as described in *Section 2.6.2*. (A) Sucrose density (g/cm ^{3}) (---), and percent recovery of loaded protein (□) and of catalase activity (□) in gradient fractions are presented. (B) Equal volumes of gradient fractions were analyzed by immunoblotting with antibodies to peroxisomal matrix (AOX, ICL, SKL, THI) and membrane (Pex16p, Pex2p) proteins and with antibodies to the cytosolic protein, G6PDH.

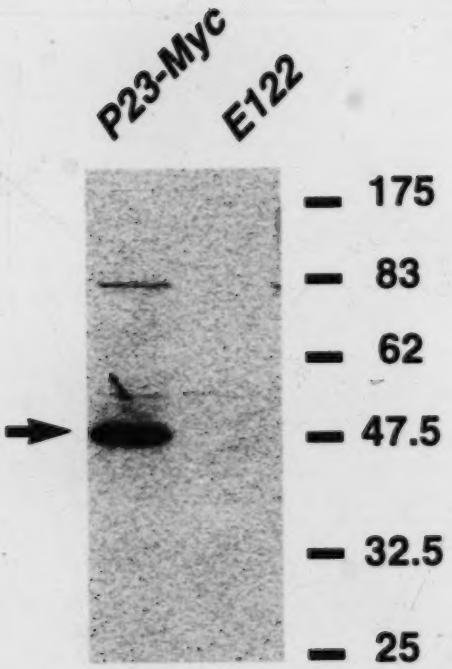

Figure 3.10. Identification of Pex23p-Myc. Strains *P23-Myc* and *E122* were grown for 16 h in YEPD medium to an OD_{600} of ~1.2 and then transferred to oleate-containing medium for 8 h. Total cell lysates were prepared, and equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-Myc (9E10) mouse monoclonal antibodies. The arrow indicates Pex23p-Myc.

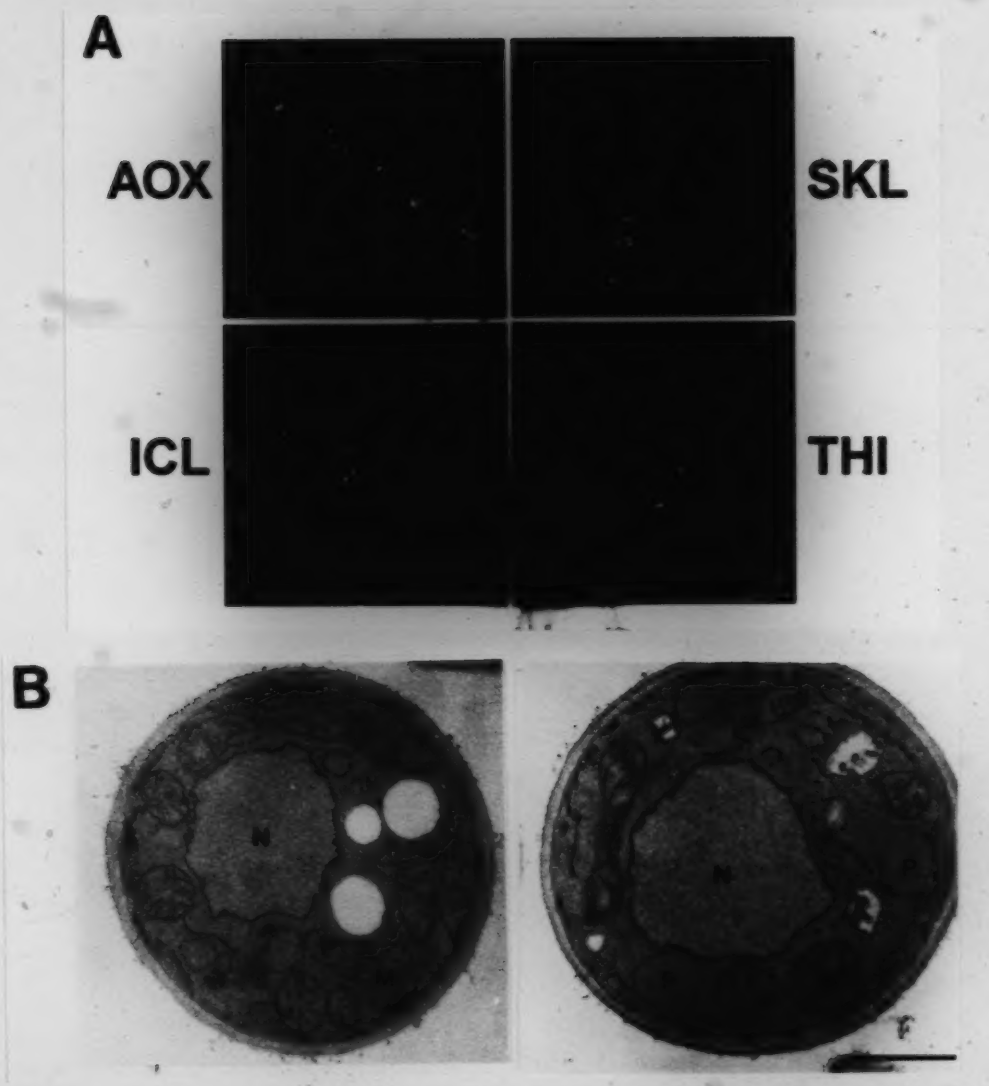

Figure 3.11. Expression of Pex23p-Myc restores peroxisome formation. Immunofluorescence (A) and electron microscopic (B) analyses of strain *P23-Myc* expressing the protein Pex23p-Myc. (A) Cells were grown in oleic acid-containing YNO medium and processed for immunofluorescence microscopy with antibodies to acyl-CoA oxidase (AOX), isocitrate lyase (ICL), the PTS1 tripeptide SKL (SKL) and thiolase (THI), as described in the legend to Figure 4. The characteristic punctate pattern of staining of peroxisomes is seen. (B) Cells were grown in YNO medium and processed for electron microscopy as described in the legend to Figure 3. Typical peroxisomes are seen. Abbreviations are as in Figure 3.6. Bar = 1 μm.

Immunoblot analysis demonstrated that Pex23p-Myc localized to fractions enriched for peroxisomes and showed essentially the same distribution across the gradient as peroxisomal matrix (AOX, ICL, THI, CAT, anti-SKL) and peroxisomal integral membrane protein (Pex2p) markers, peaking in fraction 4 of the gradient at a sucrose density of 1.21 g/cm^3 (Figure 3.12A) and well separated from fractions enriched for mitochondria (Figure 3.12B) that peaked in fraction 10 at a density of 1.17 g/cm^3.

Lysis of peroxisomes with Ti8 buffer followed by high-speed centrifugation showed Pex23p-Myc to be localized exclusively to the pellet fraction enriched for membranes, as was the peroxisomal membrane protein, Pex2p (Figure 3.13A, lane P_{Ti8}). This treatment liberated the matrix protein THI to the supernatant (Figure 3.13A, lane S_{Ti8}). Treatment of the P_{Ti8} with 0.1 M Na_2CO_3 (pH 11.5), followed by high-speed centrifugation, showed that Pex23p-Myc colocalized with Pex2p to the pellet fraction (Figure 3.13A, lane P_{CO3}), demonstrating that Pex23p-Myc is an integral protein of peroxisome membranes. This is in agreement with the sequence analysis of Pex23p predicting several potential membrane-spanning segments.

3.6 Pex23p-Myc is resistant to protease treatment in the absence of detergent

A protease protection assay was performed on isolated peroxisomes to examine the topology of Pex23p-Myc in the peroxisome membrane. Aliquots of peroxisomes were treated with increasing amounts of trypsin in the absence, or presence, of the non-ionic detergent Triton X-100. Immunoblot analysis showed no detectable degradation of Pex23p-Myc by trypsin in the absence of detergent, in a manner similar to that of the matrix protein thiolase (Figure 3.13B). In contrast, Pex2p showed cleavage by trypsin in

the absence of detergent, as has been previously demonstrated (Titorenko *et al.*, 1998). Therefore, trypsin was indeed active in the absence of detergent. Addition of increasing amounts of trypsin in the presence of Triton X-100 led to the complete degradation of Pex23p-Myc, thiolase, and Pex2p. These results are consistent with Pex23p-Myc being localized preferentially away from the cytosolic surface of the peroxisome. Since Pex23p was tagged at its C-terminus with the c-Myc epitope, our data are also consistent with the C-terminus of Pex23p being sequestered from the cytosolic face of peroxisomes.

3.7 The C-terminal -SKR of Pex23p is not necessary for its targeting to peroxisomes

The amino acid sequence for Pex23p ends with a C-terminal Ser-Lys-Arg, which is very similar to the consensus PTS1 (Ser-Lys-Leu or its variants). By tagging Pex23p at its C-terminus with the c-Myc epitope, which ends in Glu-Asp-Leu and does not resemble a PTS1 motif, the putative targeting signal would be impaired. However, Pex23p-Myc is still able to complement the mutant strain *pex23-1* (above), and is still targeted to peroxisomes. Therefore, the PTS1-like sequence is not apparently required for proper targeting of Pex23p.

3.8 Synthesis of Pex23p-Myc is induced by growth of yeast in oleic acid-containing media

Immunoblotting showed that Pex23p-Myc was barely detectable in cells grown in

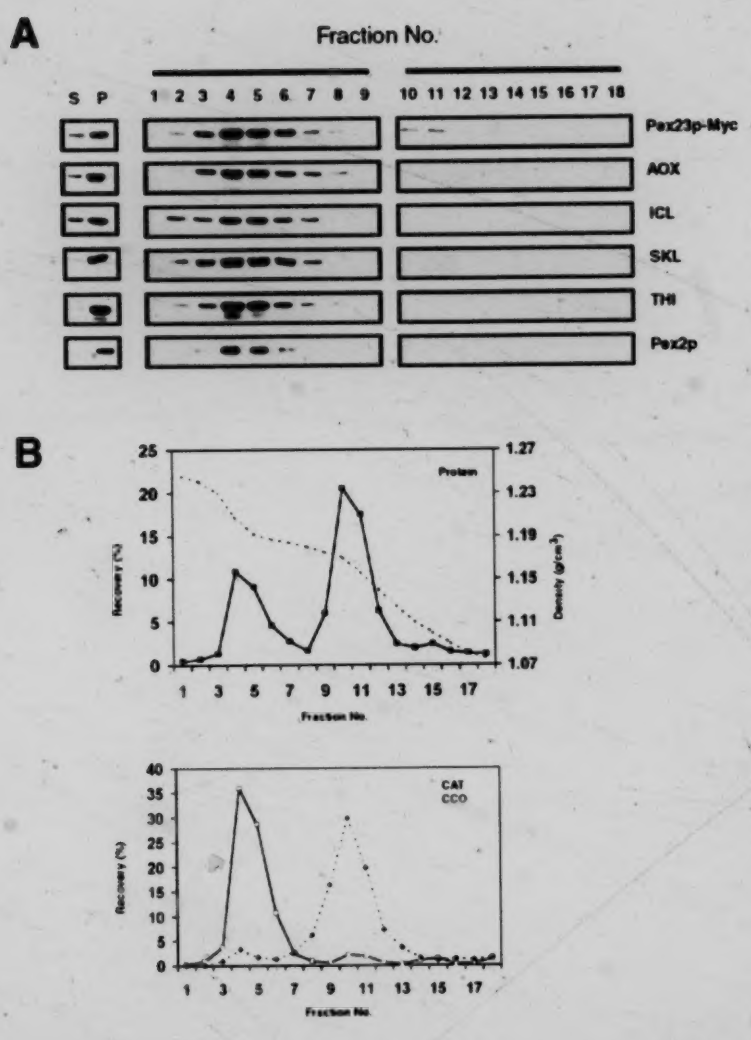

Figure 3.12. Pex23p-Myc is a peroxisomal protein. (A) Immunoblot analysis of the 20KgS (S) and 20KgP (P) fractions and of the fractions of a sucrose density gradient (numbered 1 to 18) of the 20KgP from the strain *P23-Myc* expressing Pex23p-Myc. Equal proportions of the 20KgS and 20KgP fractions, and of each of the fractions of the gradient, were analyzed by immunoblotting with antibodies to the indicated proteins. Pex23p-Myc was detected with mouse monoclonal antibody 9E10 to the c-Myc epitope. (B) Distribution of protein (– ■ –), catalase (– □ –), and cytochrome *c* oxidase (– ▲ –) across the density gradient. The dashed line in the top panel shows the density profile (g/cm^3) of the gradient.

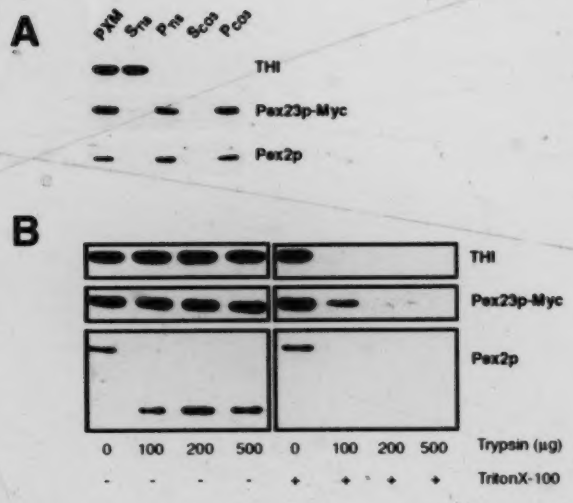

Figure 3.13. Pex23p-Myc is an integral membrane protein sequestered from the cytosolic face of the peroxisome. (A) Immunoblot analysis of whole peroxisomes (PXM) separated into pellet (P) and supernatant (S) fractions by treatment with Ti8 buffer or sodium carbonate buffer (CO3). The top blot was probed with antibodies to thiolase (THI) to detect peroxisomal matrix proteins. The middle blot was probed with mouse monoclonal antibodies to the c-Myc epitope to detect Pex23p-Myc. The bottom blot was probed with antibodies to the peroxisomal integral membrane protein Pex2p. (B) Protease protection analysis. Purified peroxisomes from the *P23-Myc* strain were incubated with increasing amounts of the protease trypsin in the absence (–) or presence (+) of the detergent Triton X-100.

glucose-containing medium, but its levels were increased significantly after cells were shifted to oleic acid-containing medium (Figure 3.14). Under these conditions, THI also showed increased levels of synthesis when cells were grown in oleic acid-containing medium, as has been reported previously (Titorenko *et al.*, 1998). In contrast, there was no change in the levels of the cytosolic enzyme, G6PDH, under the same conditions.

3.9 Morphological analysis of strain *P23TH*

Preliminary investigations into the morphological consequences of expressing Pex23p under the control of the thiolase promoter were performed. *PEX23* was cloned into the plasmid pTC3 (Section 2.4.8), which was introduced by electroporation into wild-type *E122* cells to make the strain *P23TH*. *P23TH* was grown in glucose-containing medium, as well as in peroxisome-inducing oleate medium, and was then analyzed by indirect immunofluorescence and electron microscopy.

Immunofluorescence microscopy did not reveal any differences in the locations of SKL, thiolase or AOX proteins in the *P23TH* strain as compared to the wild-type strain, nor was their any difference observed in apparent peroxisome size (Figure 3.15).

Electron microscopy of glucose-grown (Figure 3.16B) and oleate-grown (Figure 3.16A) cells showed a marked difference in peroxisome abundance due to induction, but the oleate-grown cells still showed normal peroxisomes. Interestingly, there appeared to be increased vacuolar clumping and in many cases, vacuolar membranes were seen to protrude into one another (Figure 3.16C and D). The nature or relevance of this morphological feature is unknown at this time.

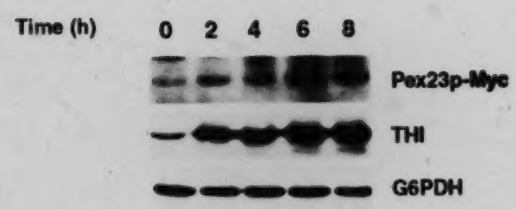

Figure 3.14. Synthesis of Pex23p-Myc is induced by growth of *Y. lipolytica* in oleic acid-containing medium. Strain *P23-Myc* was grown to an OD_{600} = 2.0 in glucose-containing YND medium (Time = 0 h) and then transferred at a dilution of 1:4 to oleic acid-containing YNO medium. Samples were taken from YNO medium at the times indicated. At each time point, equal amounts of protein of total cell lysates were analyzed by SDS-PAGE, followed by transfer to nitrocellulose and immunoblotting with mouse monoclonal antibodies to the c-Myc epitope to detect Pex23p-Myc (top panel) and with antibodies to THI (middle panel) and the cytosolic enzyme glucose-6-phosphate dehydrogenase (G6PDH) (bottom panel).

Figure 3.15. Indirect immunofluorescence labelling of strain *P23TH*. Cells transformed with *PEX23* under the control of the thiolase promoter were grown overnight in glucose-containing YND medium, shifted to YNO medium for 8 h, and processed for immunofluorescence microscopy as described in *Section 2.5.2*. Antibodies are as described in the legend to Figure 3.7

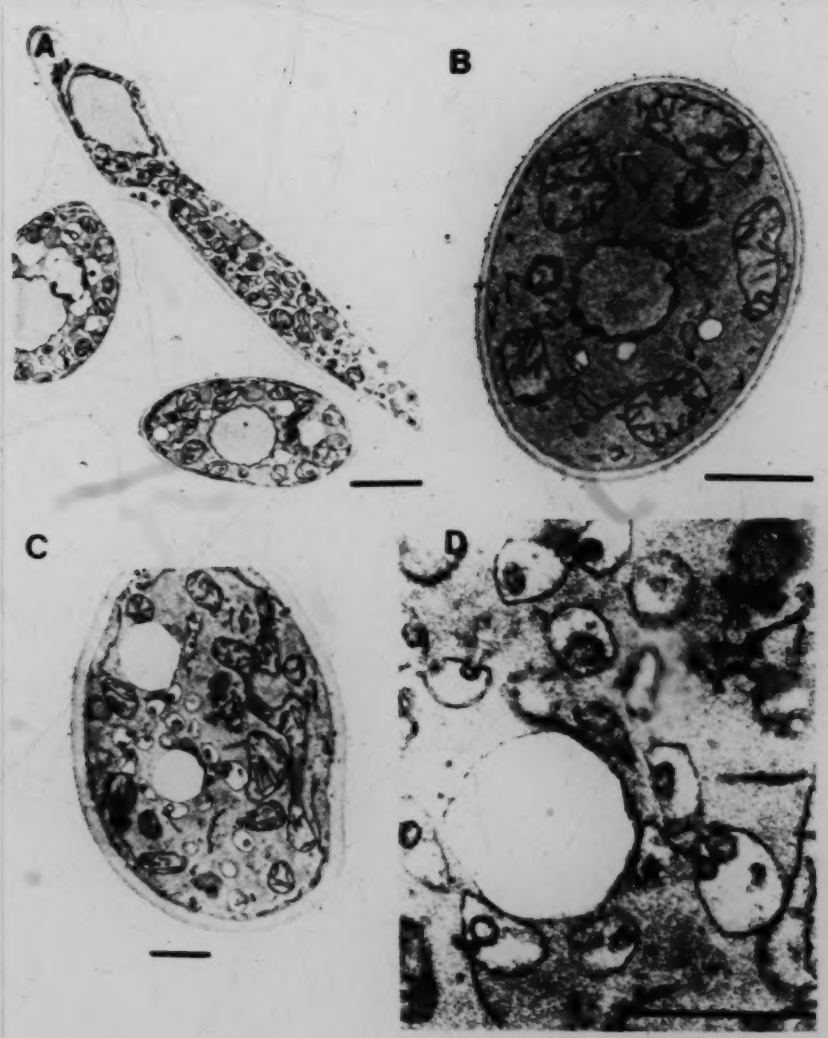

Figure 3.16. Ultrastructure of strain *P23TH*. Strain *P23TH* was grown overnight in glucose-containing YND medium (panel B) and then shifted to YNO medium for 8 h (panels A and C) and processed for electron microscopy. Compare panels A (oleate-grown) and B (glucose-grown). Peroxisomes appear normal. Arrows indicate observed vacuolar blebbing. Panel D is a higher magnification of the view in panel C. Bar = 1 μm.

4.0 Summary and Discussion

The work presented in this thesis reports the isolation of a novel *pex* mutant, *Y. lipolytica pex23-1*, and its morphological and biochemical characterization. We also report the cloning and sequencing of the *PEX23* gene and the preliminary characterization of its gene product, Pex23p.

4.1 *PEX23* is a novel gene involved in the peroxisome biogenesis pathway

pex23-1 was initially identified by its inability to use oleic acid as its sole carbon source and has been shown to be a *bona fide* peroxisome assembly mutant by its inability to correctly localize a subset of matrix proteins to peroxisomes. Complementation of the mutant phenotype of *pex23-1* with a *Y. lipolytica* genomic library identified a novel *PEX* gene, *PEX23*, of 1254 nucleotides coding for a protein of 418 amino acids. The encoded peroxin, Pex23p, has a predicted molecular weight of 47,558 Da and an overall basic charge with a pI of 9.1. Sequence analysis revealed either two (Eisenberg *et al.*, 1984; Klein *et al.*, 1985) or three (Rao and Argos, 1986) potential membrane-spanning domains.

Pex23p shows a high degree of sequence similarity with two putative *S. cerevisiae* proteins, YLR324p and YGR004p, and to a lesser extent with *S. cerevisiae* YBR168p. None of these proteins has been characterized. Possible functional redundancy amongst these three proteins may have prevented their ready identification as *PEX* genes in *S. cerevisiae* using random mutagenesis selection procedures.

4.2 The peroxin Pex23p is an integral membrane protein se questered from th cytosol

Resistance to sodium carbonate extraction indicated that Pex23p is most likely an integral membrane protein, which is in agreement with the predictions of Pex23p having several membrane-spanning domains. Topological analysis indicated that Pex23p is predominantly sequestered from the cytosolic face of the peroxisomal membrane, as shown by its protection from proteolysis in the absence of the membrane-disrupting detergent Triton X-100. Given the inconclusive nature of the total number of membrane-spanning domains, protection of any cytosolically exposed portions of Pex23p can be reconciled by their association within a complex that may mask any proteolytically accessible sites, thereby protecting them from degradation. However, there is no evidence at this time for any association of Pex23p within a protein complex.

4.3 Targeting of Pex23p to the peroxisom

Pex23p possesses a C-terminal –SKR sequence that is similar to the PTS1 consensus sequence of -SKL but which is not required for peroxisomal targeting or functioning of Pex23p. Fusion of a c-Myc epitope to the C-terminus of Pex23p, changing the terminal tripeptide from an -SKR to an -EDL, still permitted Pex23p-Myc to complement the mutant phenotype, demonstrating the expendability of this sequence for Pex23p function.

Recently, mPTS consensus sequences have been identified for several PMPs, including *Cb*PMP47 (McCammon *et al.*, 1993; Dyer *et al.*, 1996) and *Pp*Pex3p (Wiemer *et al.*, 1996). Although the individual consensus sequences and their locations often

differ, a charged stretch of basic amino acids (usually five) are an essential element (*e.g.* KIKKR in *Cb*PMP47 and RNKKK in *Pp*Pex3p). Visual inspection of the Pex23p amino acid sequence revealed a similarly charged stretch of basic amino acids (KEKKK) located at the N-terminus of Pex23p. The mPTS of *Pp*Pex3p is similarly located. This stretch of basic amino acids in Pex23p may or may not be part of an mPTS for Pex23p. This domain would be a good candidate for targeting studies using truncated stretches of Pex23p fused to reporter proteins that would ultimately reveal whether or not this sequence acts as a true mPTS.

4.4 *pex23* mutants accumulate a subpopulation of small vesicles that contain peroxisomal proteins

pex23 mutant cells grown on oleic acid-containing medium display a large number of small vesicular structures that are rarely seen in wild-type cells grown under the same conditions. They also display a proliferation of perinuclear membrane sheets upon induction in oleate-containing medium.

Immunofluorescence and subcellular fractionation data from the *pex23* mutants demonstrated that all peroxisomal matrix proteins tested, including those targeted by PTS1 (SKL and ICL) and PTS2 (thiolase), and those by an as of yet uncharacterized peroxisome targeting signal (AOX), are mislocalized to the cytosol. Significant amounts of the peroxisomal integral membrane protein Pex2p and of the peripheral membrane protein Pex16p were correctly targeted to fractions enriched for peroxisomes and mitochondria (20KgP). Flotation gradient analysis of the 20KgP fraction demonstrated the presence of very-low density, membrane-enclosed vesicles containing both

peroxisomal matrix and membrane proteins in the *pex23KOA* strain. These vesicles are different from the classical "peroxisome ghosts", which were originally defined as membranous structures containing peroxisomal membrane proteins but lacking peroxisomal matrix proteins (Santos *et al.*, 1988). It should be noted that the amount of matrix proteins contained within these vesicles is a fraction of the total peroxisomal matrix proteins produced by the cell, the majority of which are mislocalized to the cytosol. These vesicles are light, equilibrating at a density of 1.11 g/cm ^{3} in sucrose, much lighter than mitochondria which typically have a buoyant density of 1.17 g/cm ^{3} (this study) and far lighter than mature peroxisomes which have a density around 1.21 g/cm^3 (this study). This low density subpopulation of peroxisomal vesicles is similar to the low density subpopulations isolated from strains mutant for genes encoding other PMPs, including *YlPex9* (Eitzen *et al.*, 1995), *YlPex2* (Titorenko *et al.*, 1996) and *YlPex16* (Eitzen *et al.*, 1997). Whether the low density vesicles found in *pex23* mutants are peroxisomal precursors arrested in a specific stage of assembly caused by the absence of Pex23p, or are actually small peroxisomes that develop via an anomalous pathway, is unknown at present.

Studies have previously shown that the assembly of peroxisomes can occur in several temporally and spatially separated steps. Peroxisomal membrane proliferation and assembly have been shown to precede peroxisomal protein import and organellar growth (Veenhuis and Goodman, 1990; McCollum *et al.*, 1993; Titorenko *et al.*, 1996). Morphological and biochemical data demonstrating the proper targeting of the PMPs Pex2p and Pex16p in the *pex23* mutants seem to indicate that proper membrane proliferation and assembly are occurring in these mutants. There are no *de facto* criteria,

however, constituting functional membrane proliferation, and it cannot be discounted that other membrane proteins may fail to be properly targeted and/or inserted in *pex23* mutants.

4.5 Possible roles for Pex23p

The discovery that Pex23p is not required for the targeting of the PMPs Pex2p and Pex16p but is required for the targeting of all matrix proteins tested reinforces the view that separate targeting and translocation pathways exist for membrane and matrix proteins (Subramani, 1998). Indeed, initial steps in matrix protein import are shown to occur via separate pathways. PTS1-mediated delivery involves recognition by the PTS1 receptor Pex5p, PTS2-mediated delivery involves the PTS2 receptor Pex7p, and non-PTS1 and non-PTS2-mediated delivery (*e.g.* acyl-CoA oxidase) occurs by a yet uncharacterized pathway (reviewed in Subramani, 1993, 1995). The separate matrix import pathways are thought to converge at the level of the peroxisomal membrane. Evidence for this is provided by the identification of PTS receptor docking factors such as Pex14p and Pex13p. Pex14p is an integral PMP that has been shown to bind both Pex5p and Pex7p (Albertini *et al.*, 1997; Brocard *et al.*, 1997; Huhse *et al.*, 1998; Girzalsky *et al.*, 1999; Shimizu *et al.*, 1999). Pex13p is another PMP member of this docking complex that was initially identified as the docking factor for Pex5p (Elgersma *et al.*, 1996; Erdmann and Blobel, 1996, Gould *et al.*, 1996) and has recently been shown to bind Pex7p and to be necessary for the peroxisomal association of Pex14p (Girzalsky *et al.*, 1999). These peroxins, along with Pex17p, are thought to form a membrane-bound docking complex that may even function as part of a common translocation machinery

responsible for the active import of matrix proteins (Hettema *et al.*, 1999). The existence of a common translocation pathway has yet to be demonstrated experimentally.

Mislocalization of all peroxisomal matrix proteins investigated, including acyl-CoA oxidase which has neither PTS1 nor PTS2 motifs (Wang *et al.*, 1999), in the *pex23* mutant strains indicates that Pex23p may act at this level of convergence or even downstream of this point. Pex23p may act directly as part of the translocation machinery, and its absence would significantly decrease the rate of import of all matrix proteins. However, preliminary two hybrid analysis failed to detect any interaction between Pex23p and Pex5p, whose localization in *Y. lipolytica* is intraperoxisomal (Szilard *et al.*, 1995). Pex23p may also act indirectly by stabilizing the assembly of the complex while not actually participating in the translocation of proteins. The absence of the PMP Pex23p may destabilize the complex to such an extent that the small amount of matrix proteins present in the very-low density peroxisome-like vesicles represents a basal level of protein translocation across the peroxisomal membrane in the absence of a structurally competent translocon. Further study of Pex23p is essential to confidently define its functional role in peroxisome assembly.

4.6 Conclusions and future directions

The results presented in this thesis demonstrate that *YlPEX23* is an important gene whose product, Pex23p, plays a critical role in the proper assembly of peroxisomes. However, these preliminary data must be supplemented with further analysis of Pex23p before it can be placed in a functional category. Identification of interacting partners through physical (*e.g.* two-hybrid analysis, coimmunoprecipitation and cross-linking) or

genetic (*e.g.* synthetic lethality) screens would reveal much more information as to the actual function(s) of Pex23p. Investigation of the actual topology of Pex23p within the peroxisomal membrane and identification of the means by which it is targeted to that membrane are necessary. Biochemical analysis of Pex23p overexpression may also contribute to the knowledge of its functions.

Ultimately, further identification of proteins involved in peroxisome biogenesis in *Yarrowia lipolytica* as well as in other organisms and their characterization will continue to contribute to the growing body of knowledge regarding the assembly of peroxisomes and hopefully to a greater understanding of the mechanisms of organellar biogenesis as a whole.

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